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In Re Application of:
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For: A NOVEL RET-INDEPENDENT SIGNALING PATHWAY FOR GDNF

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Box ☒ Patent Application
☐ Provisional ☐ Design

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☐ continuation-in-part of prior application number
____/____.

☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

- ☐ Provisional Application Cover Sheet.
- ☒ New or Revised Specification, including pages 1 to 55 containing:
- ☒ Specification
 - ☒ Claims
 - ☒ Abstract
 - ☐ Substitute Specification, including Claims and Abstract.
- ☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.
- ☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.
- ☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.
- ☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application:
- ☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____ filed _____

- ☐ Signed Statement attached deleting inventor(s) named in the prior application.
- ☐ A Preliminary Amendment.
- ☒ **Eleven Sheets of Figures 1-9** Sheets of ☒ Formal ☐ Informal Drawings.
- ☐ Petition to Accept Photographic Drawings.
- ☐ Petition Fee
- ☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.
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- ☐ A Recordation Form Cover Sheet.
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- ☐ The prior application is assigned of record to _____.
- ☒ Priority is claimed under 35 U.S.C. § 119(e) to United States provisional Application No. 60/102,647, filed, October 1, 1998.
- ☐ An ☐ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27
- ☐ is enclosed.
- ☐ has been filed in prior application Serial No. _____ filed _____ said status is still proper and desired in present case.
- ☐ Diskette Containing DNA/Amino Acid Sequence Information.

- ☐ Statement to Support Submission of DNA/Amino Acid Sequence Information.
- ☐ The computer readable form in this application _____ is identical with that filed in Application Serial Number _____, filed _____. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.
- ☐ Information Disclosure Statement.
- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.
- ☐ A copy of Petition for Extension of Time as filed in the prior case.
- ☐ Appended Material as follows: _____
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FEE CALCULATION:

- ☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			\$75.00	\$	\$150.00	\$
DESIGN APPLICATION			\$155.00	\$	\$310.00	\$
UTILITY APPLICATIONS BASE FEE			\$380.00	\$	\$760.00	\$760.00
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	124 - 20 =	104	\$9 each	\$	\$18 each	\$1,872.00
INDEP. CLAIMS	24 - 3 =	21	\$39 each	\$	\$78 each	\$1,638.00
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			\$130	\$	\$260	\$ 260.00
ADDITIONAL FILING FEE				\$		\$
TOTAL FILING FEE DUE				\$		\$4,530.00

- ☒ A Check is enclosed in the amount of \$4,530.00.
- ☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.
- ☐ The foregoing amount due.
- ☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.
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- ☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-

identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date:

October 1, 1999



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A NOVEL RET-INDEPENDENT SIGNALING PATHWAY FOR GDNF**5 REFERENCE TO RELATED APPLICATIONS**

This application claims priority under 35 U.S.C. § 119(e) to U.S. provisional application 60/102,647 filed October 1, 1998, incorporated herein by reference in its entirety.

10 FIELD OF THE INVENTION

The present invention relates to methods for screening for agonists and antagonists of Ret-independent intracellular signaling.

BACKGROUND OF THE INVENTION

15 Glial cell line - derived neurotrophic factor (GDNF) (Lin et al., 1993), neurturin (NTN) (Kotzbauer et al., 1996), persephin (PSP) (Milbrandt et al., 1998) and a recently discovered artemin (ART) (Baloh et al., 1998) form a group of TGF- β family-related neurotrophic proteins. Studies in primary neuronal cultures, as well as in lesioned animal models, have provided evidence that GDNF is a survival factor for embryonic midbrain
20 dopaminergic neurons (Beck et al., 1995; Lin et al., 1993; Tomac et al., 1995), spinal motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), locus coeruleus noradrenergic neurons (Arenas et al., 1995), and subpopulations of peripheral sensory, sympathetic, and parasympathetic neurons (Buj-Bello et al., 1995; Trupp et al., 1995; reviewed by Airaksinen et al., 1999 and Saarma & Sariola, 1999). The pattern of
25 neurotrophic activity of GDNF is therefore promising for its potential use in the treatment of Parkinson disease, Alzheimer disease, motoneuron diseases and several other neurodegenerative diseases. The biological importance of the GDNF family is illustrated by the phenotype of GDNF null mice which display deficits in primary sensory, sympathetic and motor neurons. These mice also fail to develop kidneys and most of the enteric nervous
30 system and they die at birth (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

Despite the potential clinical importance of the GDNF receptor family, the intracellular mechanism of GDNF's action is far from understood. Generally GDNF has been thought to act through a multi-component receptor system including a glycosyl-phosphatidyl-inositol (GPI)-anchored GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) (Jing et al., 1996; Treanor et al., 1996) and a transmembrane receptor tyrosine kinase, Ret (Durbec et al., 1996; Trupp et al., 1996). GFR $\alpha 1$, lacking an intracellular domain, has originally been assessed as a binding site for GDNF, serving only in the presentation of the GFR $\alpha 1$ /GDNF complex to Ret (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1997). There is no doubt that the Ret and GPI-anchored GFR $\alpha 1$ are necessary receptors for GDNF (Cacalano et al., 1998; Enomoto et al., 1998) since mice lacking Ret, GDNF or GFR $\alpha 1$ all share a similar phenotype and die soon after birth. However, it is not known whether these GFR α proteins can evoke intracellular signals upon the action of GDNF family proteins in the absence of Ret.

Ret and GFR $\alpha 1$ expression patterns, although similar, exhibit differences in many tissues (Trupp et al., 1997; Enomoto et al., 1998; Golden et al., 1999; Kokaia et al., 1999), which may be a sign of the distinct signaling from GFR α receptors alone or in conjunction with Ret tyrosine kinase *in trans* (Yu et al., 1998). We recently showed both *in vitro* and *in vivo* (Ylikoski et al., 1998), for example, that GDNF promotes survival of postnatal cochlear sensory neurons expressing GFR $\alpha 1$ mRNA but lacking Ret mRNA. This difference in expression patterns may be a sign of distinct Ret-independent signaling triggered by activation of GRF α receptors.

The triggering of GDNF-dependent intracellular signaling in RN33B cells has also been described (PCT/US96/18197, incorporated herein by reference). RN33B cells were described therein as expressing four putative receptors for GDNF, none of which was c-Ret. Two of the receptors were later determined to be GFR $\alpha 1$ and GFR $\alpha 2$ (reported as GDNFR α and GDNFR β , respectively, U.S. Patent Application Serial No. 08/861,990, incorporated herein by reference). The mechanism of the Ret-independent signaling, however, was not known or described.

Although GPI-anchored membrane proteins have not been conclusively shown to exhibit independent intracellular signaling functions, evidence suggesting this possibility has

been increasing (Simons and Ikonen, 1997; Friedrichson and Kurzchalia, 1998; Harder et al., 1998; Varma and Mayor, 1998; Viola et al., 1999). It has been shown, for example, that GPI-anchored proteins in the immune system can mediate intracellular signaling events, such as activation of the small G-proteins, Src-type tyrosine kinases and elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$) (Green et al., 1997; Brown and London, 1998; Viola et al., 1999). GPI-anchored independent signaling has not previously been shown in cells of the nervous system, however.

The aim of the invention, therefore, is to further elucidate Ret independent intracellular signaling. We specifically address the role of GDNF-activated signaling in dorsal root ganglion (DRG) neurons isolated from Ret-null ($Ret^{-/-}$) transgenic mice (Schuchardt et al., 1994) and in other Ret-negative cell lines, for the purpose of developing a method for identifying compounds which are agonists or antagonists of Ret independent signaling.

15 SUMMARY OF THE INVENTION

The present invention provides methods for screening for compounds that are agonists or antagonists of GPI-anchored receptor mediated intracellular signaling, more specifically, $GFR\alpha 1$ -dependent, Ret-independent intracellular signaling, and methods for preventing and treating neuronal diseases comprising the use of such compounds.

20 In one aspect, the present invention relates to methods for identifying compounds which are agonists of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising incubating nervous system cells having such receptors with a test compound and determining whether intracellular signaling has been effected in the cells.

In another aspect, the present invention relates to methods for identifying compounds 25 which are antagonists of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising incubating nervous system cells having such receptors with a test compound in the presence of a sufficient amount of an agonist of such signaling, and determining whether such signaling is decreased in the cells, as compared to controls run in the absence of the compound.

In a further aspect, the present invention relates to a method for identifying compounds which are antagonists of GFR α 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR α 1 receptors, but not Ret, with a compound to be tested in the presence of a sufficient amount of an agonist of such signaling, and
5 determining whether such signaling is decreased in the cells, as compared to controls run in the absence of the compound.

In still another aspect, the present invention relates to a method for identifying compounds which are agonists of GFR α 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR α 1 receptors, but not Ret, with a compound
10 previously determined to bind GFR α 1 and determining whether the compound causes an increase in $[Ca^{2+}]_i$.

In a further aspect, the present invention relates to a method for identifying compounds which are antagonists of GFR α 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR α 1 receptors, but not Ret, with a compound
15 to be tested in the presence of a sufficient amount of an agonist of GFR α 1-dependent, Ret-independent intracellular signaling effective for increasing $[Ca^{2+}]_i$ and determining whether cells incubated with the compound have decreased $[Ca^{2+}]_i$ levels as compared with controls not incubated with the compound.

In yet another aspect, the present invention relates to a method for identifying
20 compounds which are agonists of GFR α 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR α 1 receptors, but not Ret, with a compound to be tested, preparing a cell lysate, immunoprecipitating the lysate with anti-GFR α 1 antibodies to form an immunoprecipitate, and performing assays to measure kinase phosphorylation on that immunoprecipitate.

25 In a further aspect, the present invention relates to a method for identifying compounds which are antagonists of GFR α 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR α 1 receptors, but not Ret, with a compound to be tested in the presence of a sufficient amount of an agonist of GFR α 1-dependent, Ret-independent intracellular signaling to effect said kinase phosphorylation, preparing a cell
30 lysate, immunoprecipitating that lysate with anti-GFR α 1 antibodies to form an

immunoprecipitate, and performing assays to measure kinase phosphorylation on that immunoprecipitate, then comparing the results to controls run in the absence of the compound to be tested.

5 In yet a further aspect, the invention relates to a method for identifying agonists of $GFR\alpha 1$ -dependent, Ret-independent intracellular signaling by incubating cells which express GPI-anchored $GFR\alpha 1$ receptors, but not Ret, with a compound to be tested and determining whether Src-kinase is activated.

10 In a still further aspect, the invention relates to a method for identifying antagonists of $GFR\alpha 1$ -dependent, Ret-independent intracellular signaling by incubating cells which express GPI-anchored $GFR\alpha 1$ receptors, but not Ret, with a compound to be tested and a sufficient amount of an agonist of $GFR\alpha 1$ -dependent, Ret-independent intracellular signaling to activate Src kinase and determining whether incubation with the compound has resulted in less activation of Src kinase as compared to controls not incubated with the compound.

15 In other aspects, the present invention relates to methods for effecting cellular responses in nervous system cells comprising administering an effective amount of either an agonist or antagonist of GPI-anchored intracellular signaling.

20 In yet another aspect, the present invention relates to a method for identifying agonists of intracellular signaling effected by $GFR\alpha$ receptors comprising incubating lipid rafts prepared from cells having such receptors with a test compound, and determining whether Src-type kinase is activated

25 In yet a further aspect, the present invention relates to a method for identifying antagonists of intracellular signaling effected by $GFR\alpha$ receptors comprising incubating lipid rafts prepared from cells having such receptors with a test compound in the presence of a sufficient amount of an agonist of $GFR\alpha$ -dependent signaling to activate Src-type kinase, and determining whether Src-type kinase activation is reduced in the presence of the test compound, as compared with controls run without the test compound.

30 In a further aspect, the present invention relates to methods for treating neuronal diseases comprising the administration of an agent which is an agonist or antagonist of GPI-anchored intracellular signaling.

In further aspects, the present invention relates to methods for treating neuronal diseases comprising the administration of agents which are agonists and/or antagonists of GPI-anchored intracellular signaling.

These and other aspects of the invention will become more apparent from the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 A-G depict GDNF-evoked rapid and long-lasting $[Ca^{2+}]_i$ changes in wild type mouse DRG neurons. The vertical bars depict $\Delta[Ca^{2+}]_i$ changes of 100 nM. **A.** Neurons were loaded with 10 μ M Ca-Green 1AM. Increasing concentrations of 10-100 ng/ml GDNF, applied to the bath as indicated, evoked rapid and long-lasting elevations in $[Ca^{2+}]_i$ (n=15 separate experiments, at least 3-4 neurons recorded in each experiment). **B.** 100 ng/ml GDNF, heat-inactivated at 98 °C for 15 min did not evoke changes in $[Ca^{2+}]_i$ (8 recorded cells). In these two representative recordings thapsigargin (5 μ M) (Tha) was applied at the end of the experiments to prove the functionality of the internal calcium stores. **C.** RT-PCR shows that wild type DRG neurons express Ret (284 bp fragment), GFR α 1 (746 bp fragment), and GFR α 2 (429 bp fragment) mRNA. Neurofilament light chain (NF-L, 644 bp fragment) was used as a positive control for neuronal mRNAs. H₂O lane depicts a negative control without added mRNA. The size of molecular weight markers is shown on the right. **D.** Pre-treatment with 1U/ml PI-PLC eliminates GDNF-evoked $[Ca^{2+}]_i$ elevation. **E.** Pre-treatment with 5 μ M thapsigargin abolished the effect of GDNF. **F.** Pretreatment with the PLC γ inhibitor U-73122 also resulted in abolishing of the effect of GDNF. **G.** GDNF (100 ng/ml) evoked both transient and sustained $[Ca^{2+}]_i$ elevation in E18 DRG neurons isolated from GFR α 2-negative (GFR α 2^{-/-}) mice (9 recorded neurons). In these neurons nominal removal of external Ca²⁺ (no added EGTA, about 20 μ M free Ca²⁺) led to the rapid decline of $[Ca^{2+}]_i$. In this condition addition of GDNF evoked fast, transient and often oscillatory responses in all of these cells. Re-addition of external Ca²⁺ (wash-out) evoked a rise in $[Ca^{2+}]_i$ in GFR α 2^{-/-} (9 recorded cells) as well as in wild type DRG neurons (8 recorded cells).

Figures 2 A-D depict GDNF-triggered Ca^{2+} release from the heparin-sensitive internal calcium stores in $\text{Ret}^{-/-}$ (Ret-negative) DRG neurons. **A.** Single cell RT-PCR was performed in 18 $\text{Ret}^{-/-}$ DRG neurons. Upper panel shows $\text{GFR}\alpha 1$ mRNA expression pattern in 11 cells. Cyclophilin (CP, lower panel) was used as a control. 16 out of 18 studied neurons expressed $\text{GFR}\alpha 1$ mRNA. **B.** GDNF (100 ng/ml)-evoked long-lasting Ca^{2+} elevation in $\text{Ret}^{-/-}$ DRG neurons (the two traces are representative of 41 recordings. The vertical bars depict $d[\text{Ca}^{2+}]_i$ changes of 100 nM. **C.** Pre-treatment with PI-PLC (1 U/ml; for 1 hr at 37 °C) abolished the effect of GDNF because of removal of the GPI-anchored proteins from the membrane. To control the viability of the cells at the end of experiments the neurons were treated with thapsigargin (5 μM) and were also depolarized with 50 mM K^+ . After thapsigargin or K^+ treatment $[\text{Ca}^{2+}]_i$ was transiently increased indicating that the internal Ca^{2+} stores and voltage-operated Ca^{2+} channels were functional. The vertical bars depict $d[\text{Ca}^{2+}]_i$ changes of 100 nM. **D.** In two separate experiments, part of the neurons pre-loaded with Ca-green were injected with heparin. Control neurons were injected with the solvent. Heparin injected neurons (indicated with #2, lower trace, n=3 neurons) did not significantly respond to GDNF application whereas control neurons responded with a long-lasting $[\text{Ca}^{2+}]_i$ elevation which was slowly declining during wash-out (indicated with #1, upper trace, n=3 neurons). Vertical bar depicts $d[\text{Ca}^{2+}]_i$ changes of 100 nm.

Figures 3 A-E depict GDNF-evoked calcium entry in $\text{Ret}^{-/-}$ DRG neurons. **A.** In part of the DRG neurons, application of nominally Ca^{2+} free extracellular solution (no added EGTA) resulted in a delayed transient $[\text{Ca}^{2+}]_i$ elevation possibly due to activation of capacitative calcium entry (15 recorded neurons). This $[\text{Ca}^{2+}]_i$ overshoot was not observed when calcium concentration in the nominally Ca^{2+} free external solution was clamped to about 1 nM with 2 mM EGTA (14 recorded neurons; data not shown). A return to 2 mM external Ca^{2+} (wash-out) resulted in a pronounced $[\text{Ca}^{2+}]_i$ overshoot indicating an increased membrane permeability for Ca^{2+} . Switching back to nominally Ca^{2+} free external media in the presence of GDNF (100 ng/ml) resulted in a transient elevation of $[\text{Ca}^{2+}]_i$ with significantly prolonged kinetics of $[\text{Ca}^{2+}]_i$ decline. This indicates that at the resting membrane potential GDNF can prolong capacitative calcium entry either via more profound depletion of the internal stores

or by a direct action on calcium channels in the plasma membrane. The traces are representative of 14 recordings performed in 3 independent experiments. **B.** GDNF was repeatedly applied both in the presence of the normal extracellular Ca^{2+} concentration and in the presence of nominally Ca^{2+} free external solution in $\text{Ret}^{-/-}$ DRG neurons. Application of GDNF (100 ng/ml) in the normal calcium extracellular solution evoked a typical long-lasting Ca^{2+} elevation which was reversed by wash-out. A switch to the nominally Ca^{2+} free external solution in the continuous presence of GDNF resulted in profound (and oscillatory in 5 out of 12 recordings) increase in $[\text{Ca}^{2+}]_i$ followed by slow decline in $[\text{Ca}^{2+}]_i$. Removal of GDNF (marked with arrow 1) led to a significant decline in $[\text{Ca}^{2+}]_i$. Readmission of GDNF led to quick elevation in $[\text{Ca}^{2+}]_i$ (arrows 2 and 3) (12 recorded neurons). Switching back to normal external Ca^{2+} containing solution resulted in an additional capacitative overshoot in $[\text{Ca}^{2+}]_i$.

Figure 4 A-D depict GDNF activation of $\text{GFR}\alpha 1$ -associated Src kinases, MAP kinases and CREB in $\text{Ret}^{-/-}$ DRG neurons. **A.** PP1 (10 μM), specific inhibitor of Src-family tyrosine kinases, reversibly blocked GDNF-evoked long-lasting $[\text{Ca}^{2+}]_i$ elevation in $\text{Ret}^{-/-}$ DRG neurons. The trace is representative of six recordings. **B.** Panel 1. Triton X-100 insoluble fraction from the lysates of GDNF (100 ng/ml, 5 min) - stimulated $\text{Ret}^{-/-}$ mouse DRG neurons was immunoprecipitated (IP) with $\text{GFR}\alpha 1$ antibodies. The immunoprecipitate was subjected to an *in vitro* kinase assay and revealed a major phosphorylated ~60 kD band. Panel 2. This band was not seen in the control kinase assay performed in $\text{Ret}^{-/-}$ DRG neurons either with Protein-A Sepharose alone (a) or with bFGF antibodies instead of $\text{GFR}\alpha 1$ antibodies (b). **C.** A rapid GDNF-induced phosphorylation of p42/p44 MAP kinases (upper panel) in $\text{Ret}^{-/-}$ DRG neurons. The numbers below lanes indicate the fold induction of p42 band phosphorylation relative to control. Determination of the optical density of the bands was performed using software TINA. The lower panel shows a reprobing of the same filter with anti- $\text{GFR}\alpha 1$ mAb by Western blotting (WB) and demonstrates comparable amounts of $\text{GFR}\alpha 1$ protein in all lanes (n=3 independent experiments). **D.** GDNF induced profound increase in CREB Ser-133 phosphorylation. The numbers below lanes indicate the fold induction of CREB phosphorylation relative to control. The lower panel shows the reprobing

of the same filter with anti-CREB antibodies and demonstrate comparable amount of CREB protein in all lanes.

Figures 5 A-C depict GDNF-stimulated Src-type kinases associated with GFR α 1 in the Ret

5 negative human SHEP neuroblastoma cell line. **A.** RT-PCR analysis shows that SHEP cells express only GFR α 1 (538 bp fragment), but not GFR α 2 or Ret mRNA (expected fragments of 280 and 281 bp, correspondingly). The H₂O control is as shown in Fig. 1C. **B.** Upper panel: the Triton X-100 insoluble fraction from the lysates of SHEP cells were immunoprecipitated (IP) with anti-GFR α 1 antibodies and assayed for *in vitro* kinase activity
10 as described in the Materials and Methods section. The cells were non-treated (0 min) or treated with 100 ng/ml GDNF for the time indicated. The optical density of the bands was determined using a phosphoimager and a TINA program and is presented as fold increase relative to control (GDNF non-treated cells). Lower panel: the precipitates after the kinase assay were probed by Western blotting (WB) with pan-Src antibodies, which recognize Fyn,
15 Yes and Src. **C.** Left panel (GFR α 1): the postnuclear lysate from SHEP cells non-treated (-) or pre-treated (+) with GDNF (100 ng/ml, 1min) was precipitated with GFR α 1 antibodies. Co-precipitates were assayed for kinase activity as described in Materials and Methods section. GDNF significantly increased Src type kinase activity associated with GFR α 1. These results are representative of 5 independent experiments. Right panel (Yes): SHEP
20 cells were not-treated (-) or pre-treated (+) with GDNF (100 ng/ml; 1min), the Src-related kinase p62Yes was immunoprecipitated from the whole cell lysates (without preliminary IP with anti-GFR α 1 antibody) with an anti-Yes polyclonal antibody and assayed for kinase activity. It revealed that the major GDNF-stimulated band co-migrates with Yes type kinase of about 62 kD. The samples were normalized by protein amount.

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Figures 6 A-C depict GDNF-evoked phosphorylation of MAPK, CREB and ATF-1 in the

Ret-negative SHEP neuroblastoma cell line. **A.** Addition of GDNF evoked transient and profound increase of p42/p44 MAPK phosphorylation (left panel). The numbers below lane indicate the fold induction of p42 phosphorylation relative to control. Lower panel shows the
30 re-probing of the same filter with anti-GFR α 1 antibodies and demonstrates comparable

amount of GFR α 1 protein in all lanes. The results shown are representative of four independent experiments. **B.** GDNF induced phosphorylation of p42/p44 MAPK was completely abolished by a 5-minute pre-treatment with PP2 even at the low concentration, 1 μ M (upper panel). The blot was re-probed with the anti-GFR α 1 antibodies. Lower panel shows that GFR α 1 protein was distributed equally in all lanes (n=2 experiments). **C.** GDNF treatment of SHEP cells resulted in potent induction of CREB Ser-133 phosphorylation as well as induced phosphorylation of the CREB-related protein ATF-1. The numbers below the lanes indicate the fold induction of CREB phosphorylation relative to control. The lower panel shows the re-probing of the same filter with anti-CREB antibodies (n=2 experiments).

Figures 7 A-C depict GDNF-activated Src type kinase and MAP kinase in NIH 3T3 fibroblasts stably transfected with GFR α 1. **A.** The kinase assay experiments. The major ~60 kD proteins can be co-precipitated with GFR α 1 after GDNF (100 ng/ml) pre-treatment both in Neuro2A-20 neuroblastoma cells expressing Ret (1) and in the NIH3T3 fibroblast cells (2) (both cell lines were stably transfected with GFR α 1). **B.** GDNF induced phosphorylation of p42/p44 MAPK in NIH 3T3 cells lacking Ret (upper panel; n=3 independent experiments). Pre-treatment of the cells with selective Src kinase inhibitor, PP2 (5 μ M) for the indicated time abolished the effect of GDNF. The numbers below lanes indicate fold induction of p42 MAPK phosphorylation relative to control. The lower panel shows a reprobing of the same filter with anti-GFR α 1 antibodies and demonstrates comparable amounts of GFR α 1 protein in all lanes (n=2 independent experiments). **C.** Neuro2A neuroblastoma cells expressing Ret but not endogenous GFR α 1 was treated with GDNF in the presence of a soluble GFR α 1 (GFR α 1/Fc chimeric protein; 1 μ g/ml) lacking a GPI anchor. Soluble GFR α 1 induced GDNF-dependent phosphorylation of p42/p44 MAPK. The numbers below lanes indicate fold induction of p42 MAPK phosphorylation relative to control (non-treated with GDNF).

Figures 8 A-C depict GDNF-increased PLC γ tyrosine phosphorylation in SHEP neuroblastoma cells. **A.** SHEP cells were incubated with the indicated concentrations of GDNF for 1 min, and then lysed. Tyrosine-phosphorylated proteins were

immunoprecipitated with 4G-10 anti-phosphotyrosine antibodies (α -PY) and then probed for PLC γ with anti-PLC γ antibodies by Western blotting as described in the Materials and Methods. GDNF evoked a dose-dependent increase in PLC γ tyrosine phosphorylation (n=3 experiments). The samples were normalized by protein amount. **B.** Left panel: SHEP cells were incubated with indicated concentrations of GDNF for 1 min or for 5 min. PLC γ proteins were immunoprecipitated from the lysates by anti-PLC γ antibodies. The immunocomplexes were probed for tyrosine-phosphorylated proteins with 4G-10 antibodies (α -PY) by Western blotting. Right panel: the blot was re-probed with anti-PLC γ antibodies. In all panels the numbers below lanes indicate fold induction of PLC γ phosphorylation relative to control. **C.** GDNF application did not affect the phosphorylation of JNK (n=2 experiments).

Figures 9 A-B depict a schematic representation of the proposed Ret-independent GDNF-evoked signaling pathway. **A.** The GDNF-triggered membrane signaling most probably occurs within lipid rafts, as GFR α 1 protein can be co-precipitated with Src type kinases in Triton X-100 insoluble membrane fractions. **B.** GDNF-evoked activation of GFR α 1 induces Src type kinase (in particular, pp62^{Yes} kinase in SHEP cells) activation and subsequent phosphorylation of PLC γ and MAP kinases. PLC γ activation leads to IP₃-dependent release of Ca²⁺ from internal calcium stores. Src-dependent phosphorylation of MAPK lead to its translocation to the nucleus and CREB activation..

DETAILED DESCRIPTION

An increasing amount of evidence suggests that GPI-linked proteins associated with lipid rafts (Sargiacomo, 1993; Fra et al., 1994; Casey, 1995; Simons & Ikonen, 1997; Xavier et al., 1998) are able to mediate intracellular signaling events *in vitro* and *in vivo* (Green et al., 1997; Simons and Ikonen, 1997; Mayor et al., 1998). The existence of microdomains of GPI-anchored proteins was recently shown in living cells (Varma & Mayor, 1998; Friedrichson & Kurzchalia, 1998). The GFR α proteins, as described above, are GPI-anchored.

We disclose herein that GDNF can evoke potent intracellular signaling through a Ret-independent, GPI-anchored GFR α 1-mediated pathway. We also disclose that, upon GDNF binding, GPI-anchored GFR α 1 can solely evoke the induction of PLC γ signaling pathway that is dependent on the activation of Src Family kinases and results in the

5 long-lasting sustained [Ca²⁺]_i. We further disclose that in addition to activating PLC γ and calcium signaling, GDNF activates Ret-independent Src kinase-mediated ERK1/ERK2 (MAPK) and CREB in Ret^{-/-} DRG neurons and in the different Ret-negative cell lines.

We further describe assays for compounds that are agonists or antagonists of GPI-anchored intracellular signaling based upon [Ca²⁺]_i elevation and the activation of Src
10 family kinases. We also disclose methods for identifying compounds that are agonists or antagonists of GPI-anchored intracellular signaling based upon phosphorylation of MAPK and CREB.

Whether a compound binds to GFR α 1 can be readily determined by one skilled in the art using, for example, methods for identifying compounds which bind receptors for GDNF
15 as described in U.S. Patent Application Serial No. 08/861,990, incorporated herein by reference. Alternatively, the Biacore device (Pharmacia) can be used. Src kinase activity can be determined indirectly by looking at the tyrosine phosphorylation state or kinase activity of its substrates that include, but is not limited to, p130Cas (Sakai et al., 1997) and FAK (Polte et al., 1997). Methods for determining MAPK and CREB activation are
20 described herein.

Activation of the GFR α 1-dependent, Ret-independent signaling pathways may stimulate different cellular responses than those of the Ret-dependent pathway. For instance, neuronal survival, neurite extension, enhancement of neurotransmitter synthesis, or other cellular responses to GDNF may be preferentially enhanced by one pathway over the
25 other. Agonists or antagonists of one of these signaling mechanisms may provide for more specific cellular responses than that of GDNF itself and thereby gain therapeutic advantage over GDNF. Methods for identifying agonists and antagonists of Ret-dependent intracellular signaling is described in U.S. Patent Application Serial No. 08/861,990, incorporated herein by reference. Methods to develop agonists of both signaling pathways, antagonists of both

pathways, agonists or antagonists of just one pathway, or compounds that agonize one pathway but antagonize the other pathway are contemplated herein.

GFR α 1-dependent, Ret-independent signaling may promote the survival and function of specific neuronal populations. Auditory neurons, which receive the impulses from the sensory auditory hair cells and transmit them to the brain, respond to GDNF both in vitro and in vivo. GDNF has been shown to protect neurons of the inner ear. (Tay et al., 1998; Shoji et al., 1998; and Keithley et al., 1998.) GFR α 1 is expressed on auditory neurons in the absence of Ret. Therefore, a mechanism is described herein that suggests how the Ret-independent signaling works and how this population of neurons may be supported by compounds that specifically mimic GDNF dimerization of GFR α 1 at the receptor or at the subsequent intracellular signaling events.

“A”, “an”, and “the” as used herein refer to the singular and plural.

The term “effect” as used herein means an alteration or change. An effect can be positive, such as causing an increase in some material, or negative, e.g., antagonistic or inhibiting.

The term “agonist” as used herein refers to a compound or composition that can stimulate or positively influence the intracellular signaling pathways described herein, or augment or synergize the activity of any other compound or composition thereon.

The term “antagonist” as used herein refers to a compound or composition that can inhibit, suppress, block or negatively influence the intracellular signaling pathways described herein.

The term “sufficient amount” as used herein refers to a quantity of an agent that will result in the referred to effect.

The term “bind” as used herein refers to the interaction between ligands and their receptors, the binding being of a sufficient strength and for a sufficient time to allow the detection of said binding under the conditions of the assays disclosed herein.

The term “about” in reference to a numerical value means $\pm 10\%$ of the numerical value, more preferably $\pm 5\%$, most preferably $\pm 2\%$.

The term "administration" includes but is not limited to, oral, subbuccal, transdermal, parenteral, subcutaneous and topical. A common requirement for these routes of administration is efficient and easy delivery.

As used herein, the term "effective amount," refers to the amount required to achieve an intended purpose for both prophylaxis or treatment without undesirable side effects, such as toxicity, irritation or allergic response. Although individual needs may vary, the determination of optimal ranges for effective amounts of formulations is within the skill of the art. Human doses can readily be extrapolated from animal studies (Katocs *et al.*, Chapter 27 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a formulation, which can be adjusted by one skilled in the art, will vary depending on several factors, including the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy, if required, and the nature and scope of the desired effect(s) (Nies *et al.*, Chapter 3 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996).

The term "nervous system cell" as used herein refers to all cells present in or derived from the nervous system, including, but not limited to neuronal cells, such as neurons, and non-neuronal cells, such as glial cells.

The term "transformed cell" as used herein refers to a cell that has been modified using procedures known in the art to express GFR α 1 and/or not to express Ret.

The term "neuronal disease," as used herein, means any disturbance in structure or function of any nervous system cells, from whatever cause, and shall include all abnormalities, whether originating genetically or environmentally, present congenitally or later acquired, and from any cause, whether infectious, traumatic, toxic, degenerative, inflammatory or neoplastic. This shall include any neurodegenerative or retrogressive process within one or more cells of the nervous system, including even the death of nerves, axons, or tracts of the central nervous system.

The term "cellular response" as used herein refers to, without limitation, any change in neuronal survival, neuronal plasticity, neurite extension, cell migration, or any enhancement or inhibition of neurotransmitter synthesis and/or release.

5 The term "lipid rafts," as used herein, refers to a structure of sphingolipids and cholesterol packed into moving platforms within the liquid bilayer of cell membranes, and includes the detergent insoluble, glycolipid-enriched fraction that remains after extraction with Triton X-100 or similar detergents.

The terms "GPI-anchored" or "GPI-linked" as used herein in reference to a receptor refer to a receptor that is associated with GPI.

10 The term "independent intracellular signaling" in reference to a receptor as used herein refers to a receptor that evokes intracellular signaling without requiring and/or in the absence of co-receptors.

We surprisingly found that long-lasting $[Ca^{2+}]_i$ elevations were obtained in response to GDNF (10-100 ng/ml) in DRG neurons isolated from the $Ret^{-/-}$ mice. Investigation of this
15 phenomena showed that GDNF-evoked $[Ca^{2+}]_i$ elevation persisted in nominally free extracellular Ca^{2+} solution, and can be effectively blocked by either removing GPI-linked proteins, PLC γ inhibition or by the intracellular administration of low molecular weight heparin, an antagonist of IP $_3$ -sensitive Ca^{2+} release channels on internal stores. Thus in $Ret^{-/-}$ DRG neurons GDNF triggers the release of Ca^{2+} from the internal Ca^{2+} stores perhaps via the
20 PLC γ -dependent Ca^{2+} release pathway. Presently we cannot, however, exclude the existence of another calcium release mechanism. Unfortunately, U-73122 is not a selective inhibitor of only PLC γ and heparin may also have other effects that of a competitive block of the IP $_3$ binding sites at calcium release channels. Nevertheless, both low molecular weight heparin used in this study (Thorn et al., 1993; Meyer zu Heringdorf, 1998; Takei et al., 1998 and ref.
25 thereafter) and U-73122 (Bourette et al., 1997; Davletov et al., 1998; Stam et al., 1998) are the most widely used pharmacological tools for complex investigation of PLC γ dependent signaling.

In the later phase of Ret-independent long-lasting Ca^{2+} elevation, the calcium entry through calcium release activated channels (CRAC) would play a role in the maintenance of
30 the elevated $[Ca^{2+}]_i$ due to balance refilling of intracellular Ca^{2+} stores (Sharenberg and

Kinet, 1998). Sustained elevations in $[Ca^{2+}]_i$ may lead to long term potentiation of various cellular responses, including, without limitation, major alterations in gene expression, neuronal plasticity, neurite extension, and neuron survivability, and may play an important role in such areas as learning ability, memory, epilepsy, and hearing loss, to name but a few.

- 5 Potent capacitative calcium entry aimed in re-filling of depleted calcium stores exists in DRG neurons (Usachev et al., 1999).

We disclose that GDNF can stimulate a different pattern of $[Ca^{2+}]_i$ elevation in Ret-containing wild type and $GFR\alpha 2^{-/-}$ DRG neurons via the triggering of calcium release from heparin and PLC γ -sensitive internal stores. Ret tyrosine kinase has been shown to evoke a
 10 potent PLC γ activation through a PLC γ binding site (Borrello et al., 1996). Although there is a common view that *in vivo* GDNF preferentially binds to $GFR\alpha 1$ and NTN binds to $GFR\alpha 2$, GDNF at high concentrations *in vitro* can activate $GFR\alpha 2$ and NTN can bind to $GFR\alpha 1$ (Buj-Bello, 1997; Jing et al., 1997; Klein et al., 1997; Suvanto et al., 1997; reviewed by Airaksinen et al., 1999). To study the contribution of $GFR\alpha 2$ in the action of GDNF we
 15 investigated the effect of GDNF on $[Ca^{2+}]_i$ in $GFR\alpha 2$ -negative ($GFR\alpha 2^{-/-}$) DRG neurons isolated from $GFR\alpha 2$ deficient mice (Rossi et al., 1999). We found that the GDNF-dependent $[Ca^{2+}]_i$ rise in DRG neurons was specifically dependent on $GFR\alpha 1$ activation, since GDNF evoked the same pattern of $[Ca^{2+}]_i$ elevation in $GFR\alpha 2^{-/-}$ neurons as in the wild type DRG neurons.

- 20 We also disclose that the Ret-independent GDNF-evoked intracellular signaling in neurons is much broader than activation of different patterns of Ca^{2+} elevation. In addition to the GDNF-evoked Ca^{2+} rise we detected a potent GDNF-dependent transient activation of MAPK and CREB in Ret $^{-/-}$ DRG neurons. GPI-anchored protein-coupled kinases and Src-type kinases in particular, have been shown to evoke PLC γ stimulation, $[Ca^{2+}]_i$ elevation and
 25 MAPK activation (Brown and London, 1998; Dikic et al., 1996; Finkbeiner and Greenberg, 1998; Khare et al., 1997; Luttrell et al., 1996, 1997; Thomas and Brugge, 1997). Indeed in our experiments low doses of (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) or (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (both from Calbiochem), the potent and specific inhibitors of Src-type kinases,
 30 reversibly blocked GDNF-evoked $[Ca^{2+}]_i$ elevation in the Ret-negative DRG neurons as well

as inhibited GDNF-dependent activation of MAPK in Ret-negative cell lines. Thus we wanted to investigate how GPI-anchored GFR α 1 proteins lacking of an intracellular domain can, nevertheless, couple to and activate Src and MAP kinases at the cytoplasmic side of the membrane. To explore this question we used Ret-negative cell lines, SHEP neuroblastoma cells and NIH3T3 fibroblasts stably transfected with GFR α 1 cDNA.

According to current understanding (Simons and Ikonen, 1997; Brown and London, 1998) GPI-anchored proteins, transmembrane tyrosine kinase proteins, G-proteins and acylated tyrosine kinases of the Src family all can associate with so-called lipid rafts, a structure of sphingolipids and cholesterol packed into moving platforms within the liquid bilayer (Sargiacomo et al., 1993; Simons and Ikonen, 1997; Brown and London, 1998; Luttrell et al., 1999; Viola et al., 1999). Whether or not such lipid rafts exist in DRG neurons is not known. It is also not known whether GDNF family receptors are included in the rafts. An association of Src type kinases with the inner leaflet of the raft by myristilation or palmytilation links has already been demonstrated for many cell types, however (Brown and London, 1998).

To test whether GFR α 1 can be coupled with a Src-type kinase within a raft, we performed experiments involving co-precipitation of kinases possibly coupled to the GFR α 1 protein using GFR α 1 antibodies in Triton X-100 detergent resistant membrane fractions. After Triton X-100 extraction, insoluble lipids and proteins remain in the form of detergent-insoluble glycolipid-enriched complexes(DIGs) or lipid rafts (reviewed by Simons and Ikonen, 1997). We disclose herein that Src-type kinases can be co-precipitated with GFR α 1 in DIGs from Ret^{-/-} DRG neurons as well as in the different Ret-negative but GFR α 1-expressing cell lines. In DIGs from SHEP neuroblastoma cells, GDNF evoked a potent transient activation of Src kinase. These findings indicate that activation of Src-type kinases by GDNF in Ret-negative DRG neurons and neuroblastoma cells might occur within the lipid rafts.

As in the wild type DRG neurons, GDNF evoked potent activation of p42/p44 MAPK and CREB in the different Ret-deficient cell lines. Again GDNF-evoked phosphorylation of MAPK was completely abolished with low doses of the selective Src kinase inhibitor, PP2. By immunostaining with anti-phosphorylated MAPK antibodies we

also found that phosphorylated MAPK is effectively translocated to the nucleus of SHEP cells and this translocation was modulated by PP2 (Poteriaev and Titievsky, unpublished observations). Thus GDNF-evoked Ret-independent phosphorylation of MAPK is completely dependent on the GDNF-evoked activation of a Src type kinase. Since the studies on neuronal survival showed that MAPK effectively phosphorylates CREB, it was of great interest to reveal the downstream targets of activated MAPK in Ret-negative neurons and cell lines. Indeed, we detected significant GDNF-dependent activation of CREB both in the Ret^{-/-} DRG neurons and the SHEP cells. Our results are therefore to some extent contradictory to those of Trupp et al. (1999), which found that GDNF Ret-independently activates CREB phosphorylation but not Ras/ERK pathway in RN33B cells. In addition to the detection of potent GDNF-dependent ERK1/ERK2 activation, we observed a significantly prolonged kinetic of CREB activation in SHEP cells as compare to the Trupp et al. (1999) study conducted in RN33B cells. Interestingly, we also observe a robust GDNF-dependent activation of the CREB-related protein ATF-1 in Ret-negative SHEP cells but not in the Ret^{-/-} DRG neurons. It is, therefore, possible that the noticed discrepancies between our results and those reported by Trupp et al. (1999) reflect the variance between the different type of the cells.

Src kinases and MAPK might significantly affect cell function since these kinases have been established to be crucially involved in mitogenesis, nerve-growth factor induced cell differentiation with neurite outgrowth, cell migration as well as in focal adhesion kinase (FAK) dependent cell motility (Khare et al., 1997; Thomas and Brugge, 1997). A potent MAPK activation, such as observed in our experiments, might promote neuronal survival and neuronal plasticity (reviewed by Fukunaga K. & Miyamoto E., 1998 and Impey et al., 1999). At the moment, however, we do not know the exact physiological meaning of the GDNF invoked long-lasting Ca²⁺ elevation and MAPK and CREB activation in Ret^{-/-} DRG neurons and in the cell lines. Ret-independent GDNF-evoked activation of the transcription factors of the CREB family can lead to a potent up-regulation of the gene's expression. This can result in the profound changes in neuronal plasticity (Finkbeiner et al., 1997), since it has been shown that MAPK signaling facilitates memory consolidation and long-term potentiation by promoting de novo CREB-regulated gene expression (reviewed by Impey et

al., 1999). Unfortunately, the study of the possible changes in neuronal plasticity invoked by GDNF in Ret^{-/-} or GFR α 1 deficient neurons is precluded by the early postnatal death of the knock-out animals. It would appear that DRG neurons do not use this GFR α 1-mediated signaling as a survival factor, as GDNF has also not been shown to stimulate survival of Ret^{-/-} DRG neurons in culture (unpublished observations and Taraviras et al., 1999). We recently showed, however, that GDNF promotes survival of postnatal cochlear sensory neurons, which express GFR α 1 mRNA but lack Ret mRNA (Ylikoski et al., 1998). The morphological and biological consequences which might be triggered by GDNF-evoked Ret-independent signaling remain to be elucidated, but it is clear that different neuronal populations use this signaling pathway for different purposes and with different results.

One question that remains is how a signal is passed from the outer leaflet of the raft to the inner one, or how it is passed from the outside part of the membrane to the inner one? Without intending to be limited by any theory or mechanism, we propose that there may be an unknown transmembrane protein linking GFR α 1 and Src-type protein kinase that serves as the transducer of the signal. One such adapter plasma membrane-associated protein has been found recently in DRG neurons (Lang et al., 1998). In our experiments an about 72 kD unrecognised protein was immunoprecipitated with GFR α 1 antibodies. However, the interaction of a potential adapter protein with GFR α 1 should be different from the Ret-GFR α 1 coupling. The soluble GFR α 1, capable of inducing MAPK phosphorylation in the presence of GDNF in Ret-expressed cells, was unable to evoke intracellular signaling in the Ret-negative parental NIH3T3 fibroblasts. Based on these experiments we conclude that if an adapter protein exists, it would, unlike Ret, strictly require an association with the GPI anchor of GFR α 1. Another possibility may be that enzymes activated in the rafts involved in anchor release might yield soluble phospho-oligosaccharides. These would then flip across the bilayer and may function as active second messengers in the cytosol. GDNF can also trigger lipid-lipid interaction and raft coalescence-dependent accumulation of intracellular phosphorylated proteins. Whether such signaling pathways are involved in the action of GDNF is presently unknown.

In summary, we have found that GDNF evokes [Ca²⁺]_i elevation and Ret-independent activation of Src-tyrosine kinase, PLC γ , MAPK and CREB - coupled

intracellular signaling pathways in Ret^{-/-} DRG neurons and in the Ret-negative cell lines. GDNF binds to the GPI-anchored GFR α 1 with subsequent activation of Src kinases associated with GFR α 1 followed by activation of MAPK, CREB, PLC γ and sustained elevation of [Ca²⁺]_i. The proposed signaling pathway is summarized in Fig. 9.

5

EXAMPLES

Materials and methods

Neuronal cultures

10 DRG from embryonic day 18 (E18) mice were treated with trypsin (Worthington), non-neuronal cells removed by preplating, and about 95% pure neurons cultured on poly-ornithine-laminin-coated glass coverslips in Ham's F14 medium (Imperial Laboratories) with serum substitute containing NGF, BDNF and NT-3 (all from PeproTech, 2 ng/ml each) for at least 1 day before measurement. Neurotrophins were extensively washed out
15 before GDNF application. GDNF was from PeproTech and donated by Cephalon, Inc. Ret-deficient mice (Schuchardt et al., 1994) were identified from heterozygote matings by the absence of kidneys and PCR-based genotyping. GFR α 2-deficient mice were obtained from homozygote matings (Rossi et al., 1999).

[Ca²⁺]_i measurement

20 [Ca²⁺]_i was measured using a Bio-Rad MRC-1024 confocal microscope equipped with an argon-krypton laser. The cells were loaded with 10 μ M Calcium Green-1AM (Molecular Probes), a membrane permeable Ca²⁺ dye, by 30 min incubation in serum free cell-culture media at 37 °C and 5 % CO₂. After loading, the neurons were kept at room temperature in Dulbecco's PBS solution contained 20 mM HEPES (pH = 7.4) for at least
25 20 min prior to the experiments. All compounds were applied to the bath by a peristaltic pump. 6 kD Heparin (Sigma) and Alexa 568 nm fluorescent dye (Molecular Probes) were loaded into the cells using Molecular Probes' pinocytotic influx loading reagent basically according to the manufacturer instructions. The loading did not affect neuronal viability throughout the experiments. Cell injection was performed using Eppendorf microinjection
30 system. The average intensity of fluorescence in the pre-defined ROI (Region Of Interest)

was measured on-line using TimeCourse software from BioRad. The calibration of fluorescence traces was performed *in vitro* using F_{\max} and F_{\min} values obtained with the calcium calibration buffer kit (Molecular Probes). Because the calibration solutions may not reflect the intracellular environment, the experimental data are presented as delta

5 $[Ca^{2+}]_i$ calculated using $K_d=190$ nM for Calcium Green-1.

Transgenic GFR α 2 mice

Transgenic GFR α 2 knock-out mice were produced in our laboratory. To isolate GFR α 2 genomic clones, we screened a mouse 129/Sv library (Stratagene) with a rat GFR α 2 cDNA fragment as a probe. A 6.7 kb HindIII-XbaI fragment was used to

10 construct the targeting vector. A 0.5 kb NotI-XbaI fragment of the GFR α 2 gene, containing part of the first coding exon with the translation initiation site, was replaced with a 2.0 kb cassette containing the neomycin-resistance gene (neo) driven by the PGK promoter and polyadenylation signal. R1 embryonic stem cells were electroporated with linearized plasmid and selected in G418 (250ug/ml). Resistant clones were screened by

15 Southern blot analysis using a 5' outside probe that recognizes a 7.8 kb wild-type and 5.5 kb mutant band after a BamHI digest. Positive clones were further hybridized with neo and 3' outside probes to exclude random integration of the vector. Two injected clones gave germline transmission, when the chimeras were crossed to C57BL/6J OlaHsd.

Single cell RT-PCR

20 A negative pressure was applied to the micropipette, and the whole cell was harvested under visual control. The content of the pipette was expelled into a test tube containing TrizolTM reagent lysis buffer (Gibco BRL) and 1 μ g of carrier tRNA. The total RNA was isolated and the RT-PCR reaction was performed using a TitanTM One Tube RT-PCR kit (Boehringer Mannheim) according to the manufacturer's instructions. One half of

25 the material from each neuron was used to amplify GFR α 1 mRNA. The presence of total RNA in a sample was ensured by the amplification of a 216 bp fragment of cyclophilin mRNA using the second half of the material. For cyclophilin amplification, the primers from a QuantumRNATM kit (Ambion, TX) were used. The primers used for analysis of GFR α 1 expression were as follows: 5'-GCGGCACCATGTTCTAGCC-3' (SEQ ID NO:

30 1) and 5'-CAGACTCAGGCAGTTGGGCC-3' (SEQ ID NO: 2). The primers were

designed to cross at least one intron and to exclude amplification from genomic DNA. Amplification was carried out for 45 cycles at 95°C for 45 s; 62°C for 45 s; and 72°C for 60 s. The resulting fragments were identified by Southern blotting with a ³²P-labelled cDNA insert of the mouse GFR α 1 clone and a cDNA fragment of cyclophilin. Radioactive
5 signals were detected with a Fuji Bioimage analyzer BAS 2000. No fragments were obtained from the media of cultured neurons.

RT-PCR analysis of GDNF receptors in cultured DRG neurons and SHEP neuroblastoma cell line.

Total RNA from cultured DRG neurons (approximately 200 cells) was divided into
10 three equal parts and the RT-PCR was carried out as above. The primers for GFR α 2 were: 5'-TATTGGAGCATCCATCTGGG-3' (SEQ ID NO: 3) and 5'-AGCAGTTGGGCTTCTCCTTG-3' (SEQ ID NO: 4), and for Ret they were: 5'-ATGAAAGGGTACTGACCATGG-3' (SEQ ID NO: 5) and 5'-AGGACCACACATCACTTTGAG-3' (SEQ ID NO: 6). The PCR was carried out for 40
15 cycles under the conditions indicated above. In the RT-PCR analysis of SHEP cells we used the primers for human GFR α 1, GFR α 2 and Ret. The primers sequences and PCR conditions were reported by Hishiki et al. (1998).

Immuno-complex kinase assays and immunoblotting

DRG neurons (5x10⁵), SHEP neuroblastoma cells (10⁷), Neuro2A neuroblastoma
20 cells stably transfected with GFR α 1 (10⁷) or NIH3T3 cells (NIH3T3/pBpGFR α 1) stably transfected with GFR α 1 (10⁷) were incubated in serum-free culture medium without (control) or with GDNF (GDNF-treated) for 0.5-15 min at 37°C. The cells were washed twice with cold PBS/vanadate and lysed in TX-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 1 mM
25 phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Boehringer) on ice for 1 hour. The postnuclear lysates were pre-cleared by incubation with 50 μ l of 50% Protein G-Sepharose for goat polyclonal antibodies or Protein A-Sepharose CL4B (Pharmacia) for rabbit polyclonal antibodies for 1 hour at +4°C. After removal of the beads, the supernatants were incubated with 0.4-0.5 μ g of anti-GFR α 1 or anti-Yes polyclonal
30 antibodies (Santa Cruz Biotechnology) overnight at +4°C followed by incubation with

Protein G or A-Sepharose for 2 hours. The immunocomplexes were washed twice in TX-100 lysis buffer without EDTA and twice in kinase buffer (25 mM Hepes, pH 7.4, 5 mM MgCl₂, 5 mM MnCl₂, 1 mM sodium orthovanadate). Immunocomplexes were incubated in a kinase buffer supplemented with 5-10 µCi of [γ -³²P]ATP for 20 minutes at 37°C. The samples were washed out from incorporated label and were subjected to 10% SDS-PAGE. Proteins were transferred to a Hybond ECL membrane (Amersham) using semi-dry blot apparatus (Schleicher & Schuell, Dassel, FRG). Labeled proteins were visualized with a Fuji Bioimage analyzer BAS 2000 or by autoradiography. Quantification of the optical density of the blots was performed using the TINA program.

For immunoblotting, the membranes were probed with Src-2 antibodies (Santa Cruz Biotechnology) recognizing c-Src, Fyn and Yes, followed by the secondary HRP-conjugated anti-rabbit antibodies (Sigma). The membranes were developed with ECL reagents (Amersham Life Science).

Immunoprecipitation and Western blotting of PLC γ

To determine changes in GDNF-evoked PLC γ tyrosine phosphorylation the Western blotting assays were performed as described in Khare et al. (1997). Briefly, SHEP neuroblastoma cells were pre-incubated with 1 mM sodium vanadate in serum-free medium for 30 min. at 37 °C. and then were treated at 37 °C with 1-100 ng/ml GDNF for 1 min. unless otherwise indicated. Incubation was stopped by the addition of ice-cold PBS buffer containing 1 mM sodium vanadate. Whole cell lysates (final protein concentration 0.1-0.3 mg/ml) were prepared at 4 °C in an extraction buffer (pH 7.5) containing 50 mM Tris-HCl, 137 mM NaCl, 1 mM sodium vanadate, 1 mM PMSF, 10% glycerol, 1% NP-40 and protease inhibitors cocktail (Boehringer). Phosphotyrosine-containing proteins were immunoprecipitated with 4G-10 antibodies (Upstate Biotechnology), collected with Protein A Sepharose beads and separated on the 7.5% SDS-PAGE. The proteins were transferred to Hybond-ECL nitrocellulose membrane, and PLC γ was assessed by Western blotting using anti-PLC γ antibodies (Upstate Biotechnology). In some experiments we first immunoprecipitated PLC γ from the cell lysate using anti-PLC γ antibodies and probed the filter with anti-phosphotyrosine 4G-10 antibodies as described above. In these experiments after the phosphotyrosine detection of the immunoprecipitates, the

membranes were stripped and re-probed with anti-PLC γ antibodies (Upstate Biotechnology). Membranes were developed with ECL reagents.

MAPK, JNK and CREB phosphorylation assays

- 5 To assess GDNF-dependent MAPK and CREB phosphorylation in all studied cell lines, the semiconfluent cell monolayers were starved for 3 hours in serum-free medium, and then GDNF was applied for the indicated time. For the analysis of MAPK and CREB activation in *Ret*^{-/-} animals the DRG neurons were dissected from E18 mice and maintained in NGF-containing medium for 2 hours. After this time the neurons were
- 10 deprived of NGF by placing them in NGF-free medium in the presence of anti-NGF antibodies. After 2 hr without NGF, the neurons were stimulated with GDNF. In the experiments involving Src-kinase inhibition, a Src-family kinase inhibitor PP2 was added at the indicated concentrations to the cell monolayers 5 min before GDNF application. In some experiments 1 μ g/ml of soluble GFR α 1 lacking a GPI anchor (GFR α 1/Fc chimeric protein; R&D systems) was added 5 min prior to the GDNF application and was kept in
- 15 the solution during the GDNF treatment. After stimulation, the cells were briefly washed with PBS/sodium vanadate and lysed in the buffer containing TBS, 2 mM EDTA, 1% NP-40, 1% Triton X-100, 1 mM PMSF, 1 mM Na₃VO₄ and a Complete™ protease inhibitors cocktail (Boehringer Mannheim). Total cell protein for each extract was measured by
- 20 MicroBSA (Pierce) and an equivalent amount of protein was resolved electrophoretically on 10% polyacrylamide gels. The proteins were transferred to a Hybond ECL (Amersham) membrane and the blot was probed with either MAPK (ERK1/2) or JNK Anti-Active™ pAbs (Promega) according to the manufacturer's instructions. The blots were then re-probed with GFR α 1 mAbs (Transduction Laboratories). In the CREB
- 25 phosphorylation study, Western blots were probed with the antibodies that specifically recognise the Ser-133 phosphorylated form of CREB and then re-probed with the anti-CREB antibodies (New England Biolabs).

Example 1***Effect of GDNF on $[Ca^{2+}]_i$ in Ret / GFR α 1 positive wild type and GFR α 2^{-/-} DRG neurons***

In order to investigate a possible Ret-independent GDNF signaling in neurons we followed GDNF-induced changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by confocal microscopy in Ret-expressing and Ret^{-/-} DRG neurons. Two different types of GDNF-evoked changes in $[Ca^{2+}]_i$ were observed in 60-70% of the wild type DRG neurons expressing both Ret, GFR α 1 and GFR α 2 (Fig. 1C). GDNF (10 ng/ml) induced a rapid transient increase in $[Ca^{2+}]_i$, whereas the concentrations over 10 ng/ml (10-100 ng/ml) evoked both a transient and slow long-lasting elevation in $[Ca^{2+}]_i$ (Fig. 1A) (n=15 experiments; at least 3-4 neurons were recorded in each experiment). To control the specificity of the observed GDNF-evoked long-lasting Ca^{2+} elevation, we used heat-inactivated GDNF. Heat-inactivated GDNF did not evoke any changes of basal $[Ca^{2+}]_i$ (8 recorded cells) (Fig. 1B). To monitor the functionality of the internal calcium pools at the end of the experiments we usually applied 5 μ M thapsigargin, an inhibitor of SERCA pump at intracellular calcium stores. All cells responded to the application of thapsigargin with a profound elevation in $[Ca^{2+}]_i$ (two representative recordings shown on Fig. 1B). Further control experiments were performed using cleavage of GPI-anchored proteins from the membrane with phosphatidylinositol-specific phospholipase C (PI-PLC) (7 recordings). None of the pre-treated neurons responded to the application of GDNF (100 ng/ml) (Fig. 1D). Depletion of intracellular Ca^{2+} stores with 5 μ M thapsigargin (5 recordings) or pre-treatment of neurons with the PLC γ inhibitor, U-73122 (6 recordings) also resulted in the abolishing of the effect of GDNF (Figs. 1E and 1F respectively). Consistent with the specificity of GDNF (Rossi et al., 1999) responded to GDNF with the same pattern of fast and slow kinetics of Ca^{2+} elevation as the wild type neurons (Fig. 1G) (9 recordings). Nominal removal of extracellular calcium (with no added EDTA to the extracellular media $[Ca^{2+}]_o$ was about 20 μ M) led to decline in $[Ca^{2+}]_i$. Interestingly, application of GDNF in these cells resulted in a profound rapid and oscillatory (5 out of 9 cells) $[Ca^{2+}]_i$ increase (Fig. 1D). Switching the perfusion back to the calcium-containing medium resulted in a profound calcium overshoot. Our results are consistent with a recent

report, showing that depletion of intracellular calcium stores in DRG neurons evoke a substantial calcium entry aiming to re-fill these stores (Usachev et al., 1999).

5

Example 2

Effect of GDNF on $[Ca^{2+}]_i$ in $Ret^{-/-}$, $GFR\alpha 1$ positive DRG neurons

Ret tyrosine kinase can activate cytoplasmic PLC γ through the PLC γ docking site (Borrello et al., 1996), and this could lead to IP $_3$ production and subsequent Ca $^{2+}$ release.

- 10 We therefore assessed the role of Ret in the GDNF-evoked calcium signaling using DRG neurons, isolated from $Ret^{-/-}$ mice (Schuchardt et al., 1994). In 16 out of 18 tested $Ret^{-/-}$ DRG neurons we detected $GFR\alpha 1$ mRNA (11 representative lanes shown on Fig. 2A) meaning, in agreement with a recently published observation (Natarajan et al., 1999), that $Ret^{-/-}$ neurons preserved the expression of $GFR\alpha 1$ mRNA well. Surprisingly, although we
- 15 never observed a GDNF-dependent fast $[Ca^{2+}]_i$ rise in these neurons, a long-lasting $[Ca^{2+}]_i$ elevation was recorded in response to 10-100 ng/ml GDNF in 60-70% of the neurons (Fig. 2B) (n=21 experiments, 41 out of 61 neurons responded). In the control experiments DRG neurons did not respond with $[Ca^{2+}]_i$ elevations to changes of the superfusion solutions, brief switch on/off of the peristaltic pump, or prolonged application of heat-
- 20 inactivated GDNF (100 ng/ml) (3 experiments, data not shown; the experimental conditions were identical to experiments with wild type DRG neurons, see Fig. 1B). In addition the prolonged application of GDNF did not affect $[Ca^{2+}]_i$ in $Ret^{-/-}$ neurons pre-treated with PI-PLC (Fig. 2C, 3 recordings). As in the experiments with wild type DRG neurons, the viability of the neurons was verified either by depletion of intracellular stores
- 25 with thapsigargin (5 μ M) or by neuronal depolarization evoked by 50 mM external K $^{+}$ (Fig. 2C).

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Example 3***GDNF triggers Ca^{2+} release from the internal stores in Ret negative DRG neurons***

Activation of $\text{PLC}\gamma$ apparently leads to IP_3 production and subsequent Ca^{2+} release from IP_3 sensitive stores. To block IP_3 receptors on internal Ca^{2+} stores, we either injected

5 DRG neurons with an intracellular-like solution containing 6kD heparin, a competitive antagonist of the IP_3 binding sites at the IP_3 -sensitive Ca^{2+} release channels (Berridge, 1998) or loaded heparin into the cells using a pinocytotic loading reagent in a mixture with Alexa 568 fluorescent dye (n=2 experiments). In the heparin injected neurons, GDNF did not evoke an increase in $[\text{Ca}^{2+}]_i$ in comparison to the saline injected control

10 neurons (Fig. 2D). Neurons loaded by pinocytosis with heparin and the Alexa dye replied with $[\text{Ca}^{2+}]_i$ elevation in response to both thapsigargin and to high extracellular K^+ -evoked depolarization. They, however, did not respond either to 100 nM GDNF or to 10 μM ATP (data not shown). The GDNF-evoked long-lasting $[\text{Ca}^{2+}]_i$ elevation was completely abolished by pre-treatment of $\text{Ret}^{-/-}$ neurons with U-73122, an inhibitor of $\text{PLC}\gamma$ (n=3

15 experiments, data not shown). Taken together, these results indicate that in Ret-negative DRG neurons GDNF can trigger Ca^{2+} release from IP_3 -sensitive internal calcium stores via the $\text{GFR}\alpha 1/\text{PLC}\gamma$ coupled pathway.

Example 4***Effect of GDNF on calcium entry***

We hypothesized that in addition to the GDNF-evoked release of sequestered Ca^{2+} , entry of extracellular Ca^{2+} would participate in the maintenance of the elevated Ca^{2+} level observed after stimulation with GDNF. Furthermore, it has recently been shown that depletion of intracellular calcium stores in DRG neurons results in a substantial

25 capacitative calcium entry (Usachev et al., 1999). In 15 out of 24 $\text{Ret}^{-/-}$ neurons the switch to nominally Ca^{2+} free external solution ($[\text{Ca}^{2+}]_o$ about 20 μM Ca^{2+} ; the gradient of calcium over the plasma membrane was still about 200-fold under these conditions) resulted in pronounced Ca^{2+} overshoot (Fig. 3A). Since this overshoot was never observed in the cells bathed in an external solution containing 2 mM EGTA ($[\text{Ca}^{2+}]_o$ about 1 nM;

30 n=14 recordings, data not shown) we suggest that the perfusion of some cells with

5 nominally calcium free solution resulted in a rapid depletion of very labile calcium pools and concomitant capacitative entry of calcium. The refilling of intracellular stores at 20 μ M external calcium was not complete, since re-addition of normal (2 mM calcium containing) extracellular solution resulted in an additional large calcium overshoot. Re-application of GDNF in the presence of low extracellular calcium (Fig. 3A) (traces are representative of 15 recordings) evoked a massive $[Ca^{2+}]_i$ overshoot and substantially slower decay kinetics, compared with control application of nominally calcium free extracellular solution only (Fig. 3A). We concluded that GDNF prolonged capacitative calcium entry either via additional depletion of intracellular stores or via a direct effect on the plasma membrane calcium release activated channels (CRAC). In another set of experiments we tested if the prolonged decay of calcium entry is dependent on the direct action of GDNF on CRAC. In these experiments we removed GDNF from the nominally calcium free extracellular media at the middle of calcium overshoot. Representative traces of 12 recordings performed in *Ret*^{-/-} DRG neurons in three separate experiments are shown on Fig. 3B. Application of GDNF (100 ng/ml) in normal calcium-contained extracellular solution resulted in the usual long-lasting $[Ca^{2+}]_i$ elevation interrupted with a wash-out period. Switching to nominally calcium free (about 20 μ M free calcium-containing) extracellular solution resulted in a large calcium overshoot, similar to that shown in Fig. 3A. During the $[Ca^{2+}]_i$ decay GDNF was removed from the external media (indicated on Fig. 3B with arrow 1). This resulted in rapid decline of $[Ca^{2+}]_i$ suggesting that GDNF may have a direct regulatory effect on the plasma membrane CRAC, rather than an indirect effect mediated via depletion of calcium from internal stores. Re-addition of GDNF (100 ng/ml; indicated with arrows 2 and 3) in the nominally free calcium solution resulted in a profound increase in $[Ca^{2+}]_i$ (Fig. 3B; 12 recorded neurons).

Example 5

*GDNF activates a GFR α 1-coupled kinase, MAP kinases and cAMP response element binding protein (CREB) in *Ret*^{-/-} DRG neurons*

Since the effect of GDNF on $[Ca^{2+}]_i$ in *Ret*-deficient DRG neurons is specifically dependent on the presence of a GPI-anchored receptor, we focused on a signaling pathway

linked to Ret-independent GDNF-evoked activation of GFR α 1. GPI-anchored proteins have been shown to be localized to lipid rafts and directly associated with Src family kinases (Berridge, 1998; Harder et al., 1998). There is much evidence suggesting that Src family tyrosine kinases can use PLC γ as a substrate (Khare et al., 1997; Berridge, 1998; Harder et al., 1998). We found that GDNF-evoked long-lasting Ca²⁺ elevation in Ret^{-/-} neurons was significantly and reversibly inhibited by low doses of selective Src kinase inhibitor PP1 (Fig. 4A, 9 recorded neurons). *In vitro* kinase assay on anti-GFR α 1-precipitated Triton X-100 insoluble lysate from GDNF-stimulated Ret^{-/-} DRG neurons revealed a major phosphoprotein of approximately 60 kD (Fig. 4B), that corresponds to the Mr of several Src-type tyrosine kinases (p59 Fyn, pp60 Src and p62 Yes). These ~60 kD bands were absent in the control immunoprecipitation experiments (Fig. 4B).

We further checked the possibility of whether the activation of a GFR α 1-coupled kinase would lead to phosphorylation of the serine/threonine kinases ERK1 and ERK2 (MAPK) in the absence of Ret, bearing in mind that Src kinases might activate MAPK (Dikic et al., 1996). Using antibodies which recognize phosphorylated MAPK we found that in DRG neurons isolated from E18 Ret^{-/-} mice application of GDNF (100 ng/ml) evokes several-fold rapid activation of MAPK (n=3 experiments, Fig. 4C). Exposure of neurons to neurotrophins, such as BDNF, activates the Ras/ERK/pp90 ribosomal S6 kinase pathway that culminates in CREB phosphorylation (Finkbeiner et al., 1997). We found that CREB phosphorylation in Ser-133 increased substantially already at 5 min after GDNF treatment in Ret^{-/-} DRG neurons (Fig. 4D).

Example 6

GDNF stimulates GFR α 1-coupled p62Yes type kinase in SHEP neuroblastoma cells

We used SHEP human neuroblastoma cells line for further exploration of GDNF-dependent non-Ret signaling. SHEP cells lack Ret mRNA but express GFR α 1 mRNA (Fig.5A) and an ample amount of GFR α 1 protein (data not shown). On average in these cells, GDNF (100 ng/ml) evoked about a twelve-fold time-dependent increase of a Src-type kinase activity (Fig. 5B) as detected in co-precipitates with anti-GFR α 1 antibodies from Triton X-100 insoluble fractions using a phosphoimager and a TINA program. To

identify the particular Src-type kinase, activated by GDNF, we precipitated cell lysate with anti-Yes antibodies and performed the kinase assay thereafter. A major GDNF-stimulated kinase co-precipitated with GFR α 1 co-migrate with p62Yes kinase (Fig. 5C). Another protein with Mr of about 72-75 kD was also co-precipitated with GFR α 1 antibodies. The nature of this protein is unknown.

Example 7

10 *GDNF activates ERK1/ERK2, CREB and CREB-related protein ATF-1 in SHEP cells.*

In SHEP cells application of GDNF (100 ng/ml) evoked a similar rapid MAPK activation as in Ret^{-/-} DRG neurons (n=3 experiments, Fig. 6A, compare with Fig. 4C). This fast pattern of MAPK activation in Ret^{-/-} E18 DRG neurons and in SHEP neuroblastoma cells is different from the long-lasting elevation of MAPK phosphorylation observed in Ret/GFR α 1 expressing cells (Trupp et al., 1999). The GDNF-dependent phosphorylation of MAPK was almost completely blocked by Src-type kinase inhibitor, PP2, already at a low concentration (1 μ M) (Fig. 6B, n=2 separate experiments). To exclude the possibility that PP2 affected other than Src type kinases, we checked the effect of PP2 on NGF-mediated MAPK activation in PC12 cells. PP2 (0.1 – 10 μ M) did not affect NGF-dependent activation of MAPK in PC12 (data not shown).

We further investigated whether GDNF affects phosphorylation of CREB and we found that GDNF potently induced phosphorylation of CREB in Ser-133 site (Fig. 6C, n=2 experiments). Interestingly, GDNF also induced phosphorylation of the CREB-related protein ATF-1 (Fig. 6C). The dynamics of GDNF-induced Ret-independent CREB and ATF-1 phosphorylation was similar to that of GDNF-induced MAPK phosphorylation.

Example 8***GDNF induces activation of ERK1/ERK2 in NIH 3T3 fibroblasts via Src type kinases.***

NIH 3T3 fibroblasts stably transfected with GFR α 1 (NIH3T3/pBpGFR α 1) do not express Ret and were therefore used as a non-neuronal cell line for investigating the GDNF-evoked Ret-independent signaling. Src-type kinases can be also co-precipitated with GFR α 1 antibodies in these cells (n=2 experiments; Fig. 7A). GDNF (100 ng/ml) again reproducibly increased phosphorylation of MAPK in 3T3NIH/pBpGFR α 1 fibroblasts (n=3 experiments, Fig. 7B), although the MAPK activation was delayed in comparison with a rapid activation in Ret^{-/-} DRG neurons and in SHEP neuroblastoma cells. Application of PP2 (5 μ M) again eliminated GDNF-evoked MAPK phosphorylation in 3T3NIH/pBpGFR α 1 cells (Fig. 7B, right panel, n=2 experiments). In parental NIH3T3 fibroblasts lacking both Ret and GFR α 1 the application of GDNF (100 ng/ml) either alone or together with a soluble GFR α 1 (GFR α 1/Fc lacking GPI anchor; 1 μ g/ml) did not evoke any MAPK phosphorylation (data not shown). In the presence of GDNF the soluble GFR α 1 was able to phosphorylate MAPK in Neuro2A neuroblastoma cells lacking endogenous GFR α 1 but expressing Ret (Fig. 7C).

Example 9***GDNF transiently activates PLC γ but not JNK in SHEP cells.***

We investigated further whether GDNF can trigger PLC γ activation in SHEP cells. Treatment of SHEP neuroblastoma cells with GDNF (1-100 ng/ml) significantly increased PLC γ tyrosine phosphorylation (n=3 experiments, Fig. 8A). The effect was fast, with a significant increase at 1 min, and a return to basal tyrosine phosphorylation levels within 5 min (Fig. 8B). It has been shown that activation of endogenous Ret in PC12 cells as well as activation of transiently or stably expressed Ret in COS-1 cells or NIH3T3 fibroblasts leads to the phosphorylation of c-Jun NH2-terminal protein kinase (JNK) (Chiariello et al., 1998). In our experiments GDNF (100 ng/ml) did not evoke JNK activation in SHEP cells lacking Ret (n=2 experiments; Fig. 8C).

The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

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WHAT IS CLAIMED IS:

1. A method for identifying a compound that is an agonist of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising (i) incubating said nervous system cells having GPI-anchored receptors with a test compound and (ii) determining whether intracellular signaling has been effected in said cells.
5
2. The method of Claim 1, wherein said nervous system cells express GFR α receptors, but not Ret receptors.
3. The method of Claim 2, wherein said GFR α receptors are GFR α 1 receptors.
10
4. The method of Claim 1, wherein said nervous system cells are DRG neurons.
5. The method of Claim 4, wherein said DRG neurons are Ret (-/-).
6. The method of Claim 1, wherein said nervous system cells are neuroblastoma cells.
15
7. The method of Claim 1, wherein said intracellular signaling is measured as an increase in intracellular Ca²⁺ concentration as compared to controls not incubated with said compound.
8. The method of Claim 7 further comprising determining that said test compound binds to said GPI-anchored receptors.
20
9. The method of Claim 1, wherein said intracellular signaling is measured as kinase activation.
10. The method of Claim 9, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the cell lysate with an anti-

GPI-anchored receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.

11. The method of Claim 10, wherein said antibody is anti-GFR α 1.

5 12. The method of Claim 9, wherein said kinase is a Src-type kinase.

13. The method of Claim 12, wherein activation of Src-type kinase is measured as activation of MAPK.

14. The method of claim 12, wherein activation of Src-type kinase is measured as activation of CREB.

10 15. The method of claim 12, wherein activation of Src-type kinase is measured as PLC γ activation.

15 16. A method for identifying a compound that is an antagonist of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising (i) incubating said nervous system cells having GPI-anchored receptors with a test compound in the presence of a sufficient amount of an agonist of said intracellular signaling to effect intracellular signaling, and (iii) comparing the results to controls not incubated with said compound.

17. The method of Claim 16, wherein said nervous system cells express GFR α receptors.

20 18. The method of Claim 17, wherein said GFR α receptors are GFR α 1 receptors.

19. The method of Claim 16, wherein said nervous system cells are DRG neurons

20. The method of Claim 19, wherein said DRG neurons are Ret (-/-).

21. The method of Claim 16, wherein said nervous system cells are neuroblastoma cells.
22. The method of Claim 16, wherein the intracellular signaling being measured is an increase intracellular Ca^{2+} concentration.
- 5 23. The method of Claim 16, wherein the intracellular signaling being measured is kinase activation.
24. The method of Claim 23, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the cell lysate with an anti-GPI-anchored receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.
- 10 25. The method of Claim 24, wherein said antibody is anti-GFR α 1.
26. The method of Claim 23, wherein said kinase is a Src-type kinase.
27. The method of Claim 26, wherein said Src-type kinase activation is measured as activation of MAPK.
- 15 28. The method of claim 26, wherein said Src-type kinase activation is measured as CREB activation.
29. The method of claim 26, wherein said Src-type kinase activation is measured as PLC γ activation.
- 20 30. A method for identifying a compound that is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells that express GFR α 1 receptor, but not Ret receptor, with a test compound and (ii) determining whether intracellular signaling has been effected in said cells.

31. The method of Claim 30, wherein said intracellular signaling is measured as an increase in intracellular Ca^{2+} concentration as compared to controls not incubated with said compound.

5 32. The method of Claim 31 further comprising determining that said test compound binds to said GPI-anchored receptors.

33. The method of Claim 30, wherein said intracellular signaling is measured as kinase activation.

10 34. The method of Claim 33, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the detergent insoluble fraction of the cell lysate with an anti-GFR α 1 receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.

15 35. The method of Claim 33, wherein said kinase is a Src-type kinase.

36. The method of Claim 33, wherein activation of Src-type kinase is measured as activation of MAPK.

37. The method of claim 33, wherein activation of Src-type kinase is measured as activation of CREB.

20 38. The method of claim 33, wherein activation of Src-type kinase is measured as PLC γ activation.

39. A method for identifying a compound that is an antagonist of GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells that express GFR α 1 receptor, but not Ret receptor, with a test compound in the presence of a sufficient amount of an agonist of said intracellular signaling

to effect intracellular signaling, and (iii) comparing the results to controls not incubated with said compound.

40. The method of Claim 39, wherein said agonist is GDNF.

5 41. The method of Claim 39, wherein said intracellular signaling is measured as an increase in intracellular Ca^{2+} concentration as compared to controls not incubated with said compound.

42. The method of Claim 39 further comprising determining that said test compound binds to said GPI-anchored receptors.

10 43. The method of Claim 39, wherein said intracellular signaling is measured as kinase activation.

15 44. The method of Claim 43, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the detergent insoluble fraction of the cell lysate with an anti-GFR α 1 receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.

45. The method of Claim 43, wherein said kinase is a Src-type kinase.

46. The method of Claim 43, wherein activation of Src-type kinase is measured as activation of MAPK.

20 47. The method of claim 43, wherein activation of Src-type kinase is measured as activation of CREB.

48. The method of claim 43, wherein activation of Src-type kinase is measured as PLC γ activation.

49. A method for identifying a compound which is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR α 1 receptor, but not Ret receptor, with a test compound (ii) determining whether an increase in intracellular Ca²⁺ concentration is effected in said cells as compared to controls not incubated with said compound.
50. The method of Claim 49 further comprising determining that said test compound binds to GFR α 1 receptors.
51. The method of Claim 49, wherein said cells are DRG Ret (-/-) neurons.
52. The method of Claim 49, wherein said cells are transformed cells.
53. The method of Claim 49, wherein said cells are neuroblastoma cells.
54. A method for identifying a compound which is an antagonist of GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR α 1 receptors, but not Ret receptors, with a compound to be tested in the presence of a sufficient amount of an agonist of GFR α 1-dependent, Ret-independent intracellular signaling to cause an increase in intracellular Ca²⁺ concentration, and (ii) determining whether a decrease in intracellular Ca²⁺ concentration is effected, as compared with controls performed without said compound to be tested.
55. The method of Claim 54, wherein said agonist is GDNF.
56. The method of Claim 54, wherein said cells are DRG Ret (-/-) neurons.
57. The method of Claim 54, wherein said cells are transformed cells.
58. The method of Claim 54, wherein said cells are neuroblastoma cells.
59. A method for identifying a compound which is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating

cells which express GFR α 1, but not Ret, with the compound to be tested, (ii) preparing a cell lysate, (iii) immunoprecipitating the detergent insoluble fraction of the cell lysate with anti-GFR α 1 antibodies to form an immunoprecipitate, and (iv) performing an assay for measuring kinase phosphorylation on said immunoprecipitate.

60. The method of Claim 59, wherein said cells are DRG Ret (-/-) neurons.

61. The method of Claim 59, wherein said cells are transformed cells.

62. The method of Claim 59 wherein said cells are neuroblastoma cells.

63. A method for identifying a compound which is an antagonist of the GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR α 1, but not Ret, with the compound to be tested in the presence of a sufficient amount of an agonist of said intracellular signaling to effect kinase phosphorylation ii) preparing a cell lysate, (iii) immunoprecipitating the detergent insoluble fraction of the cell lysate with anti-GFR α 1 antibodies to form an immunoprecipitate, (iv) performing an assay for measuring kinase phosphorylation on said immunoprecipitate, and (v) comparing the results of said assay to those achieved in control experiments performed in the absence of said compound to be tested.

64. The method of Claim 63 wherein said agonist is GDNF.

65. The method of Claim 63, wherein said cells are DRG Ret (-/-) neurons.

66. The method of Claim 63, wherein said cells are transformed cells.

67. The method of Claim 63, wherein said cells are neuroblastoma cells.

68. A method for identifying a compound which is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR α 1, but not Ret, with a compound to be tested, and (ii)

determining whether activation of Src-type kinase is effected, as compared with controls not incubated with said compound.

69. The method of Claim 68, wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src, and Yes.

5 70. The method of Claim 68, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.

71. The method of Claim 68, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.

72. The method of Claim 68, wherein said cells are DRG Ret (-/-) neurons.

10 73. The method of Claim 68, wherein said cells are transformed cells.

74. The method of Claims 68, wherein said cells are neuroblastoma cells.

15 75. A method for identifying a compound which is an antagonist of the GFR α 1-dependent, Ret-independent intracellular signaling pathway comprising (i) incubating cells which express GFR α 1, but not Ret, with a compound to be tested in the presence of a sufficient amount of an agonist of said pathway to cause activation of Src-type kinase and (ii) determining whether said compound effects a decrease in Src-type kinase activation, as compared with controls not incubated with said compound.

20 76. The method of Claim 75 wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src and Yes.

77. The method of Claim 75, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.

78. The method of Claim 75, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.

79. The method of Claim 75, wherein said agonist is GDNF.
80. The method of Claim 75, wherein said cells are DRG Ret (-/-) neurons.
81. The method of Claim 75, wherein said cells are transformed cells.
82. The method of Claim 75, wherein said cells are neuroblastoma cells.

5 83. A method for identifying a compound which is an agonist of intracellular signaling effected by GFR α receptors comprising (i) incubating lipid rafts prepared from cells having GFR α receptors with said compound and (ii) determining whether Src-type kinase is activated as compared to controls not incubated with said compound.

10 84. The method of Claim 83 wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src and Yes.

85. The method of Claim 83, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.

15 86. The method of Claim 83, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.

87. A method for identifying a compound which is an antagonist of intracellular signaling effected by GFR α receptors comprising (i) incubating lipid rafts prepared from cells having GFR α receptors with said compound in the presence of a sufficient amount of an agonist of the GFR α -dependent, Ret-independent intracellular signaling pathway to activate Src-type kinases and (ii) comparing the results to control experiments performed in the absence of said compound.

20

88. The method of Claim 87 wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src and Yes.

89. The method of Claim 87, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.
90. The method of Claim 87, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.
- 5 91. The method of Claim 87, wherein said agonist is GDNF.
92. A method for effecting a cellular response in nervous system cells comprising administering an effective amount of an agonist or antagonist of GPI-anchored intracellular signaling.
- 10 93. The method of Claim 92, wherein said nervous system cells are motor neurons.
94. The method of Claim 92, wherein said nervous system cells are sensory neurons.
95. The method of Claim 92, wherein said sensory neurons are auditory neurons.
- 15 96. The method of Claim 92, wherein said nervous system cells are glial cells.
97. The method of claims 92-97 wherein said agonist or antagonist of GPI-anchored, intracellular signaling is an agonist or antagonist of GFR α 1-dependent, Ret-independent intracellular signaling.
- 20 98. A method for treating neuronal disease comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.

99. The method of Claim 98, wherein said agonist of GPI-anchored intracellular signaling is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling.

5 100. A method for treating neuronal disease comprising administering an effective amount of an agent which is an antagonist of GPI-anchored intracellular signaling.

101. The method of Claim 100, wherein said antagonist of GPI-anchored, intracellular signaling is an antagonist of GFR α 1-dependent, Ret-independent intracellular signaling.

10 102. A method for treating neuronal disease comprising administering an effective amount of an agent that is an agonist of both Ret-dependent and GPI-anchored, Ret-independent intracellular signaling.

15 103. The method of Claim 102, wherein said agonist of GPI-anchored, Ret-independent intracellular signaling is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling.

104. A method for treating neuronal disease comprising administering an effective amount of an agent that is an antagonist of both Ret-dependent and GPI-anchored, Ret-independent intracellular signaling.

20 105. The method of Claim 104, wherein said antagonist of GPI-anchored, Ret-independent intracellular signaling is an antagonist of GFR α 1-dependent, Ret-independent intracellular signaling.

25 106. A method for treating neuronal disease comprising administering an effective amount of an agent or agents that are agonists of Ret-dependent intracellular signaling and antagonists of GPI-anchored, Ret-independent intracellular signaling.

107. The method of Claim 106, wherein said antagonists of GPI-anchored, Ret independent intracellular signaling are antagonists of GFR α 1-dependent, Ret-independent intracellular signaling.

5 108. A method for treating neuronal disease comprising administering an effective amount of an agent or agents that are antagonists of Ret-dependent intracellular signaling and agonists of GPI-anchored, Ret-independent intracellular signaling.

10 109. The method of Claim 108, wherein said agonists of GPI-anchored, Ret independent intracellular signaling are agonists of GFR α 1-dependent, Ret-independent intracellular signaling.

110. A method for protecting auditory neurons comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.

15 111. A method for preventing memory loss comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.

112. A method of improving learning ability comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.

20 113. A method for treating epilepsy comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.

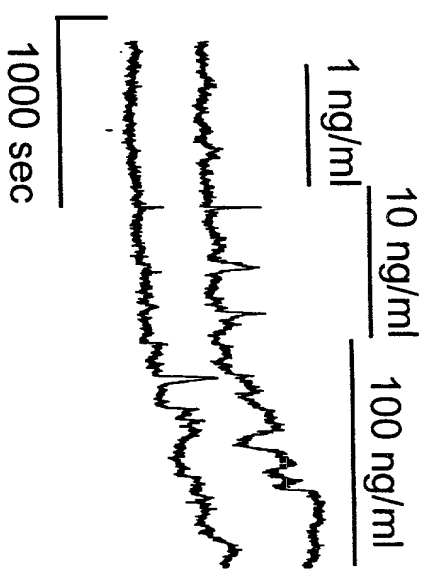
114. A method for treating Parkinson's Disease comprising administering an effective amount of an agent which is an agonist of GPI-anchored, intracellular signaling.

115. The method of Claims 110-114 wherein said agonist of GPI-anchored, intracellular signaling is an agonist of GFR α 1-dependent, Ret-independent intracellular signaling.

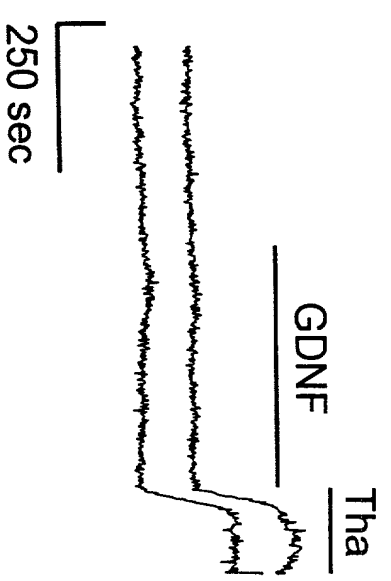
ABSTRACT

5 Methods for screening for agonists and antagonists of GPI-anchored independent intracellular signaling resulting in $[Ca^{2+}]_i$ elevation, ERK1, ERK2 and CREB phosphorylation, and Src family kinase activation are described, as well as methods for treatment involving administration of agonists/antagonists of such signaling.

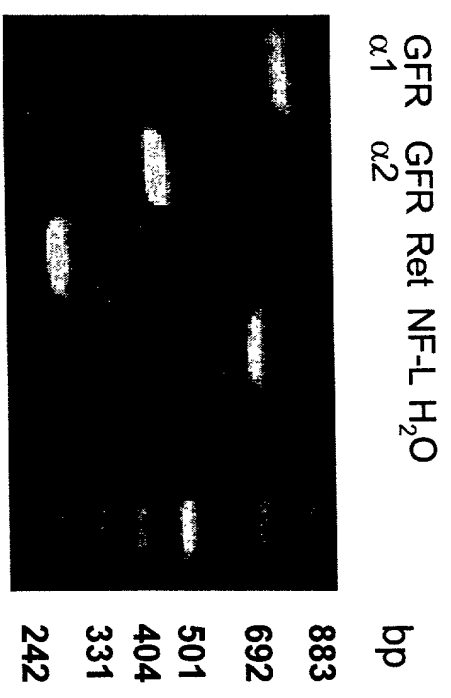
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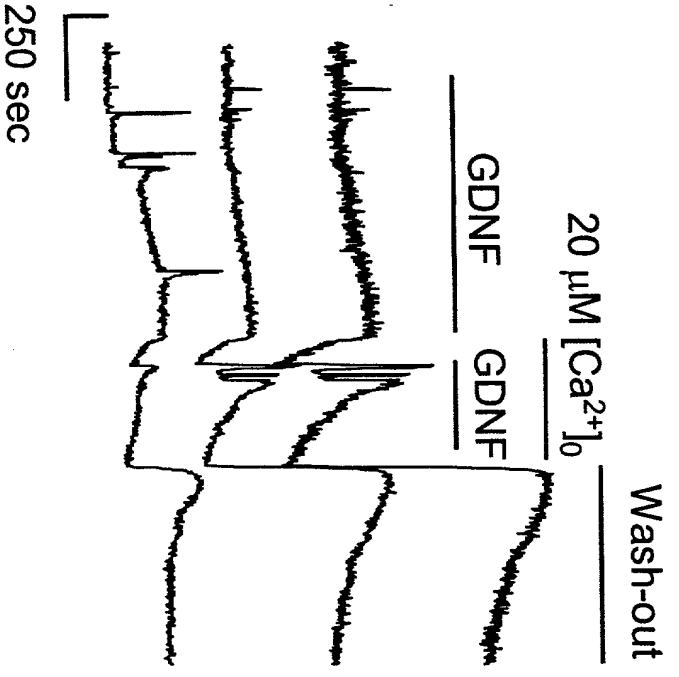
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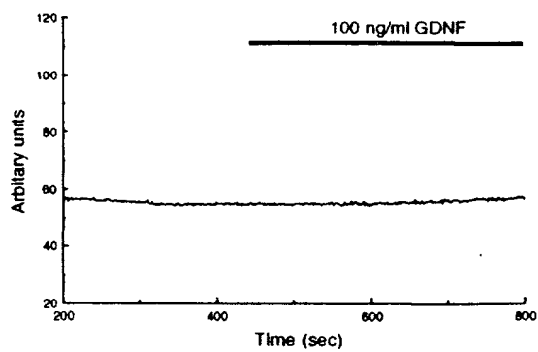
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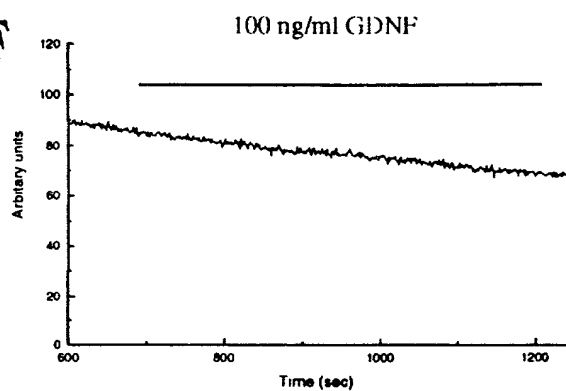
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Fig.1; Titievsky et al.

D



F



E

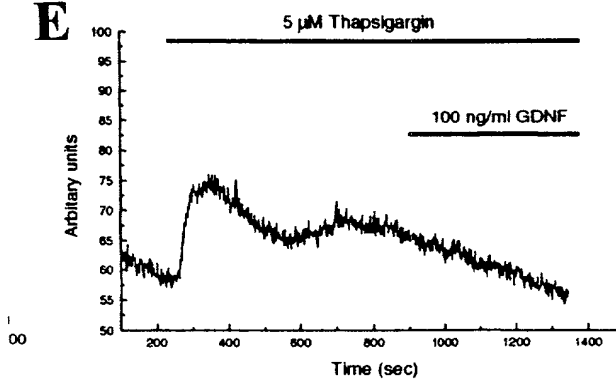
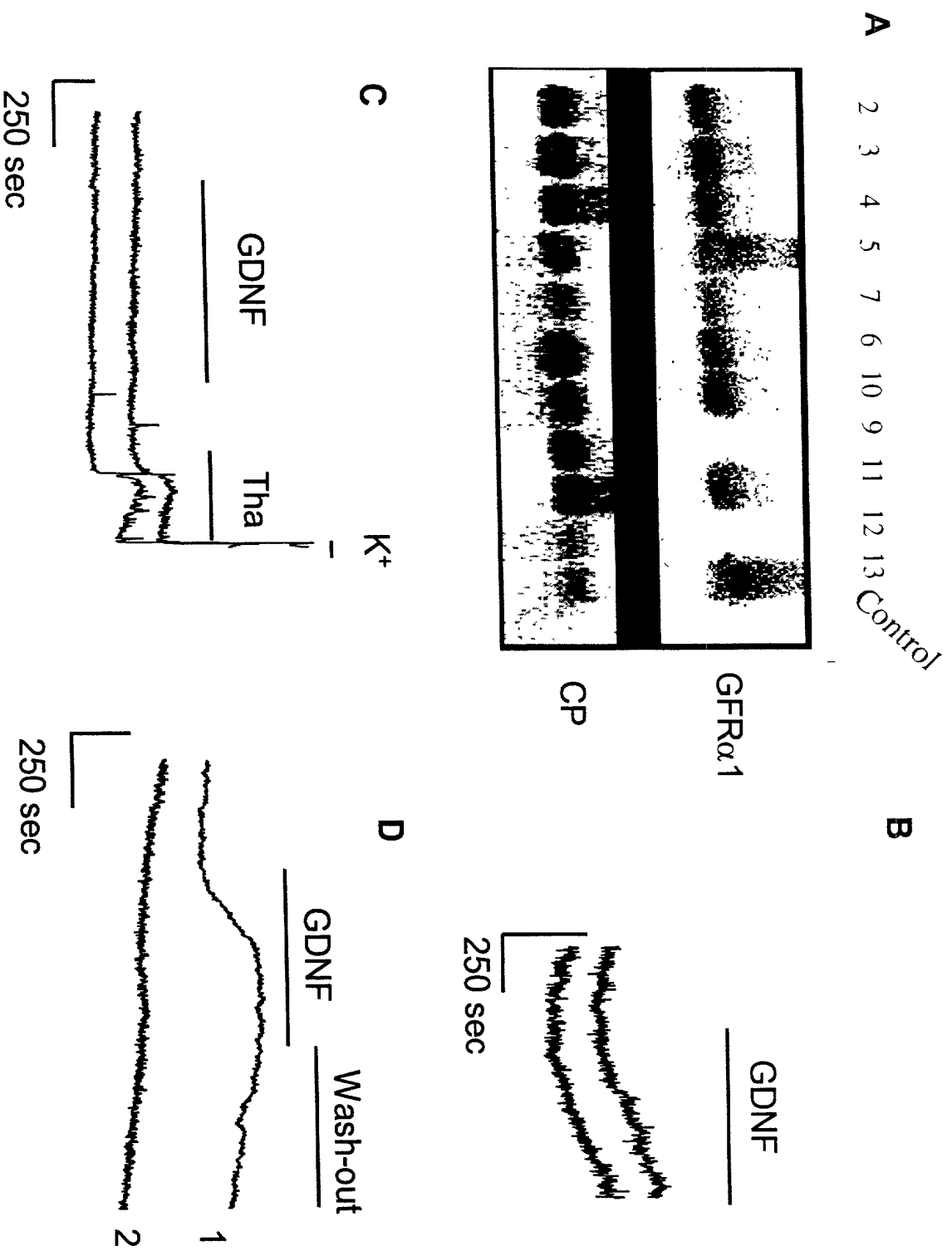


Fig. 1; Titievsky et al.



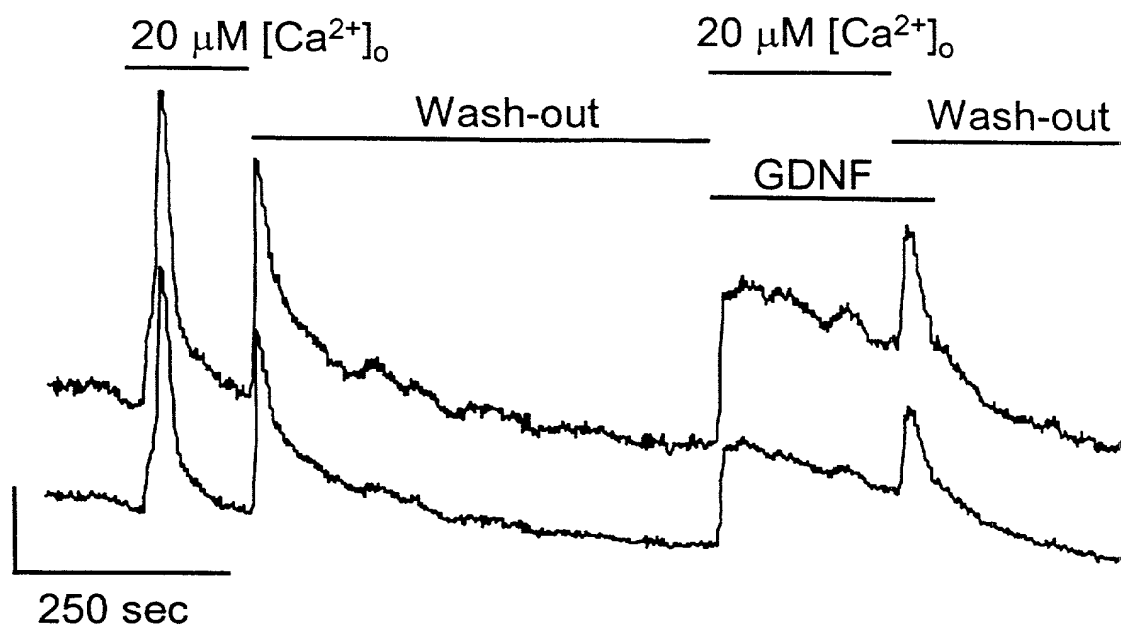
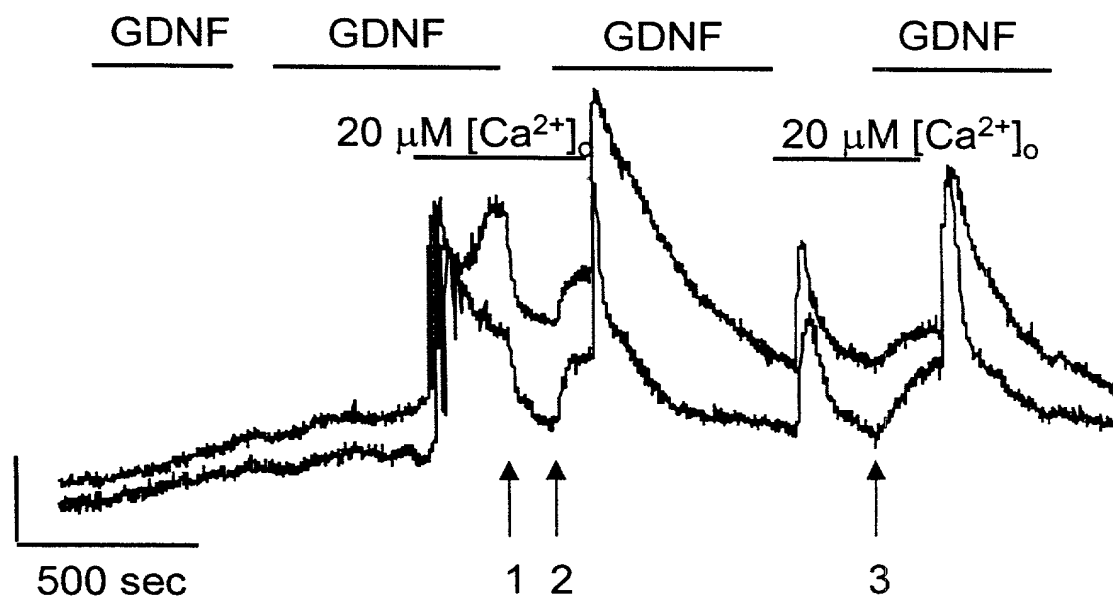
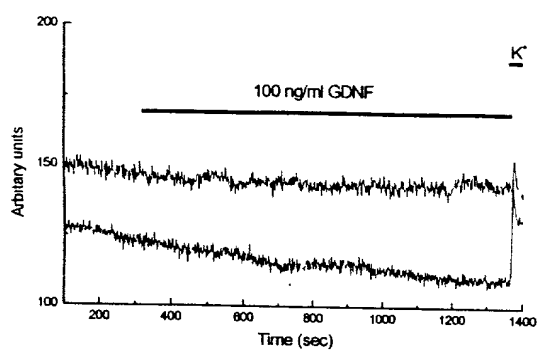
A**B**

Fig.3; Titievsky et al.

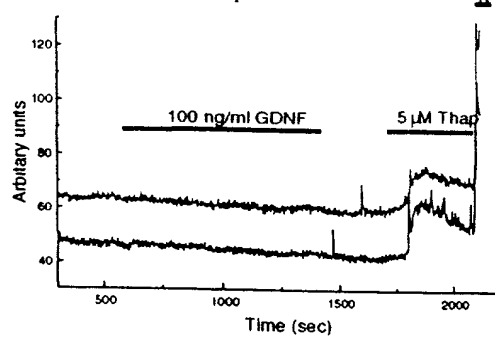
C

U-73122 pre-treated



D

PI-PLC pre-treatment



E

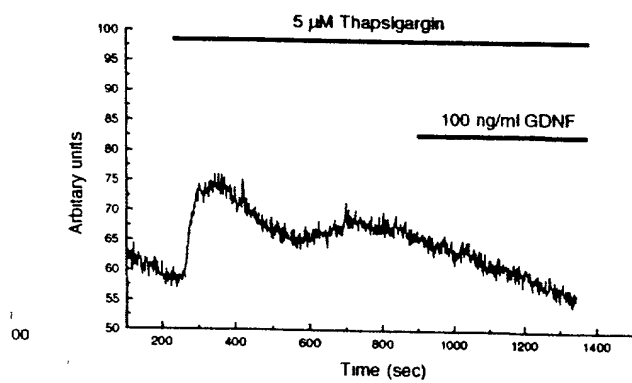


Fig. 3; Titievsky et al.

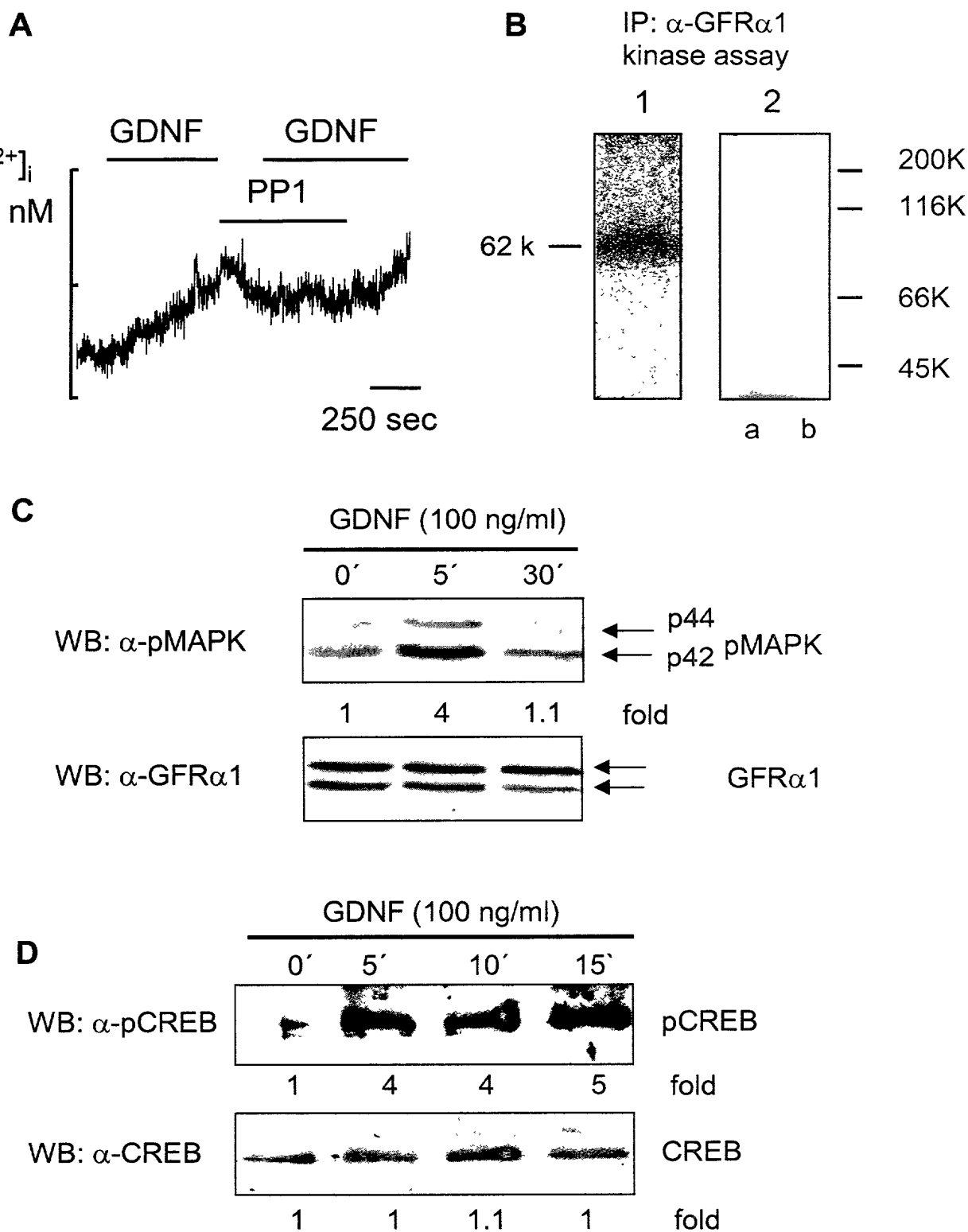
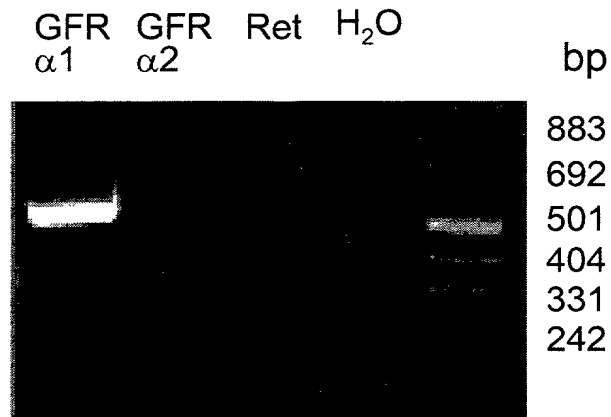
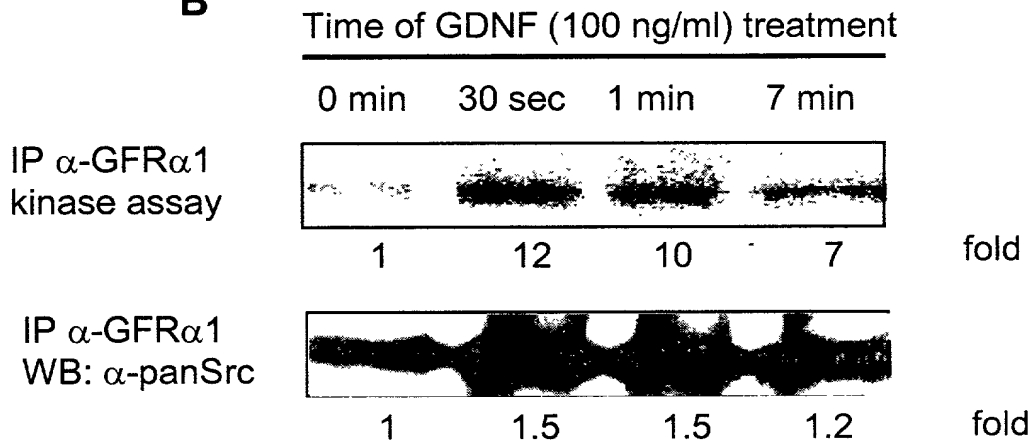


Fig.4; Titievsky et al.

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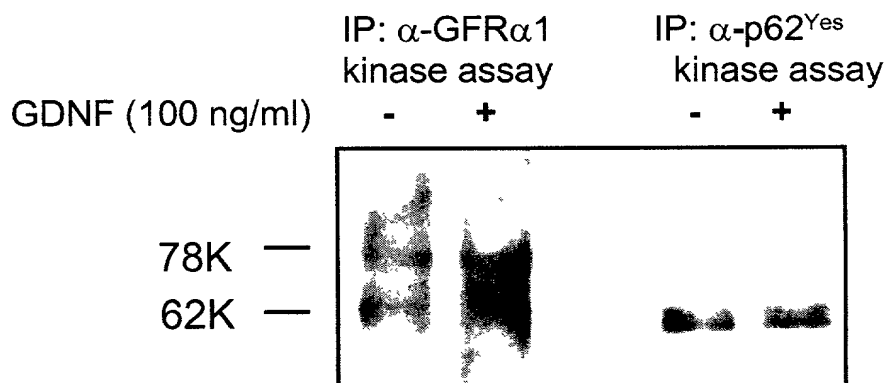
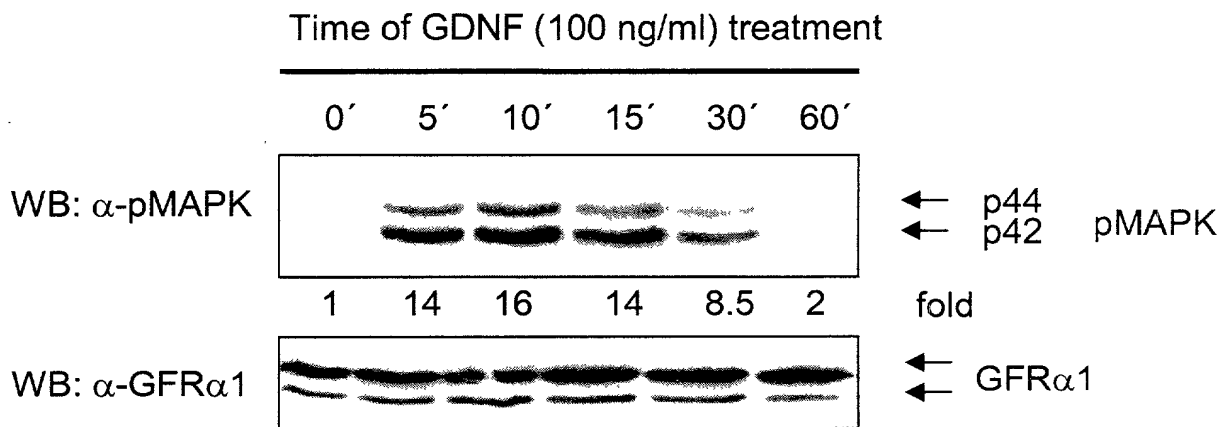
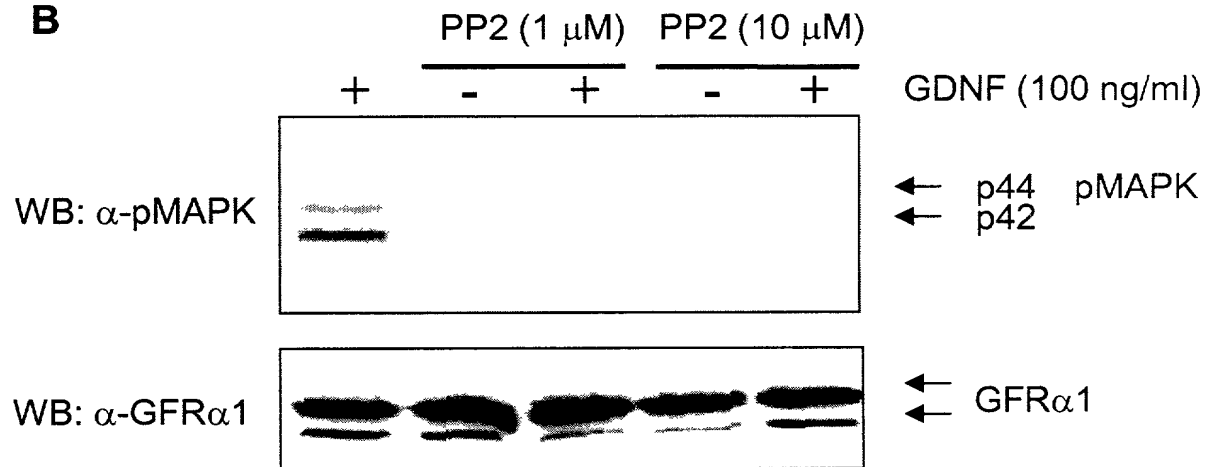


Fig.5; Titievsky et al.

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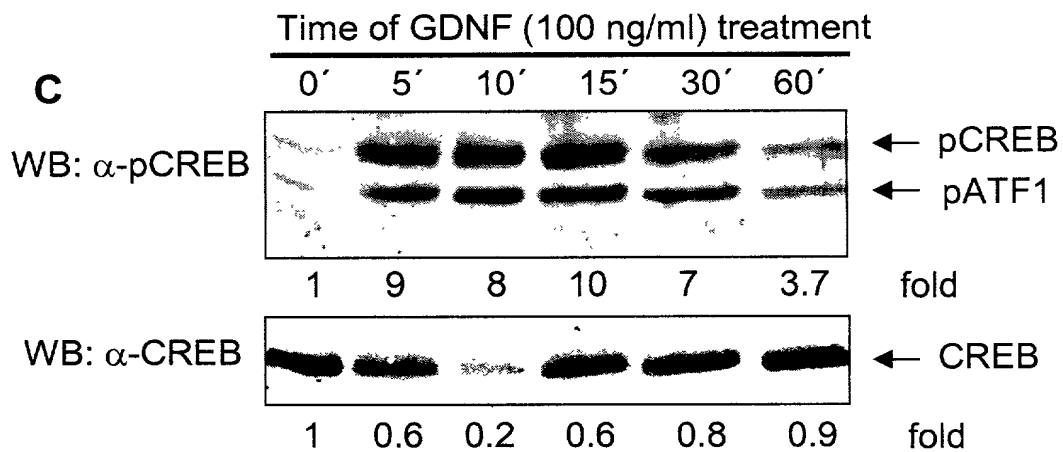
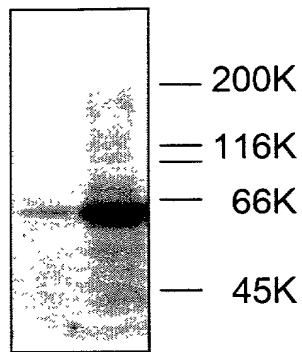


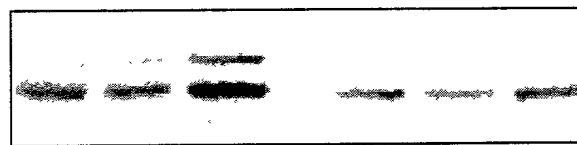
Fig.6 Titievsky et al.

AIP: α -GFR α 1
kinase assay

1 2

B

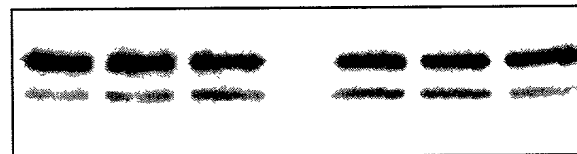
- - - + + + PP2 (5 μ M)
0' 5' 30' 0' 5' 30' GDNF (100 ng/ml)

WB: α -pMAPK

← p44
← p42

fold

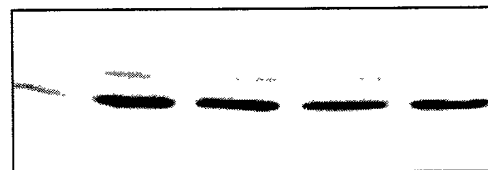
1 1 3 1 1 1.1

WB: α -GFR α 1

← GFR α 1

CGFR α 1/Fc (soluble GFR α 1)

0' 5' 15' 30' 60' GDNF (100 ng/ml)

WB: α -pMAPK

← p44
← p42

fold

1 3.7 3.3 3.3 3

Fig.7; Titievsky et al.

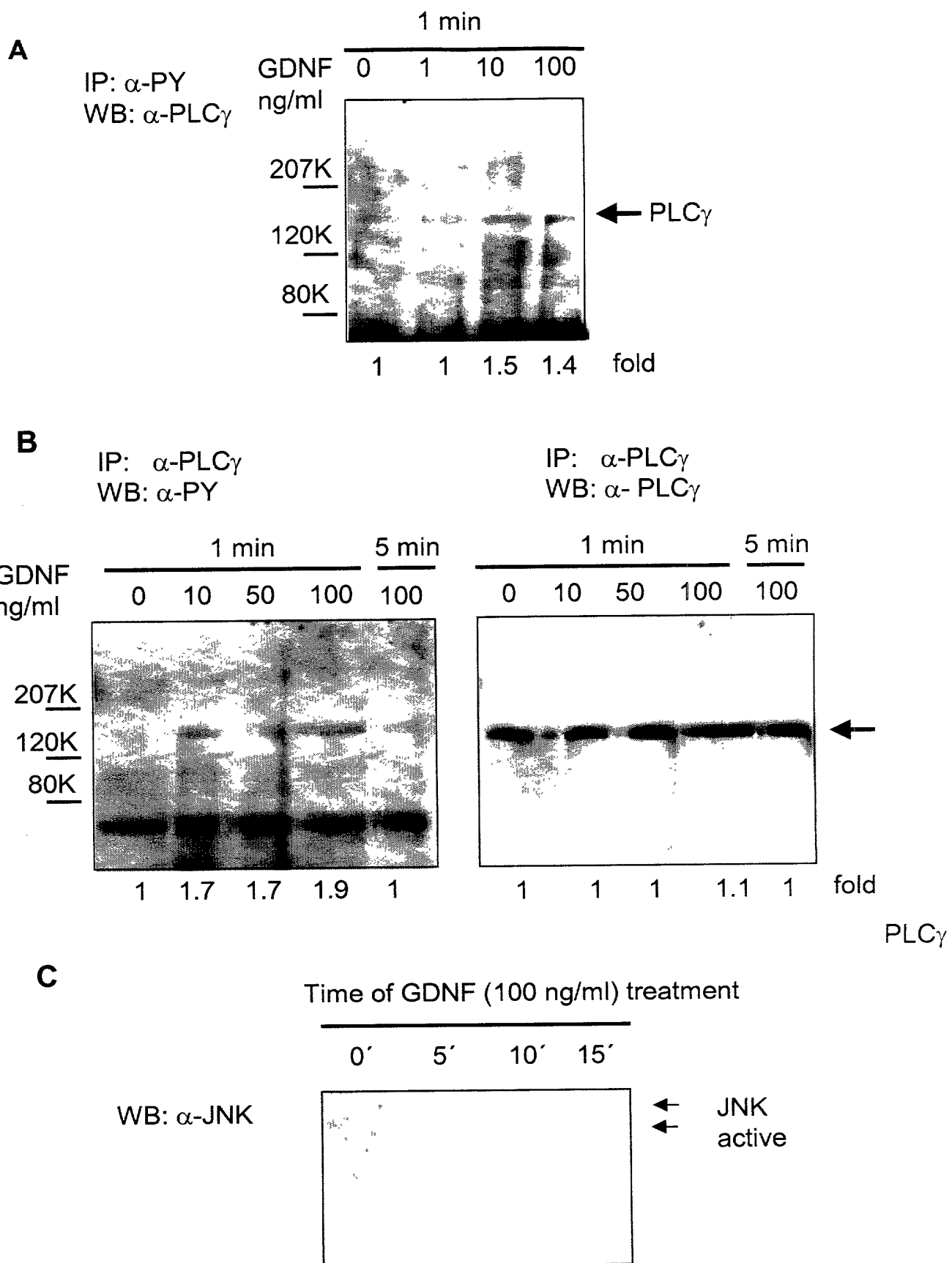
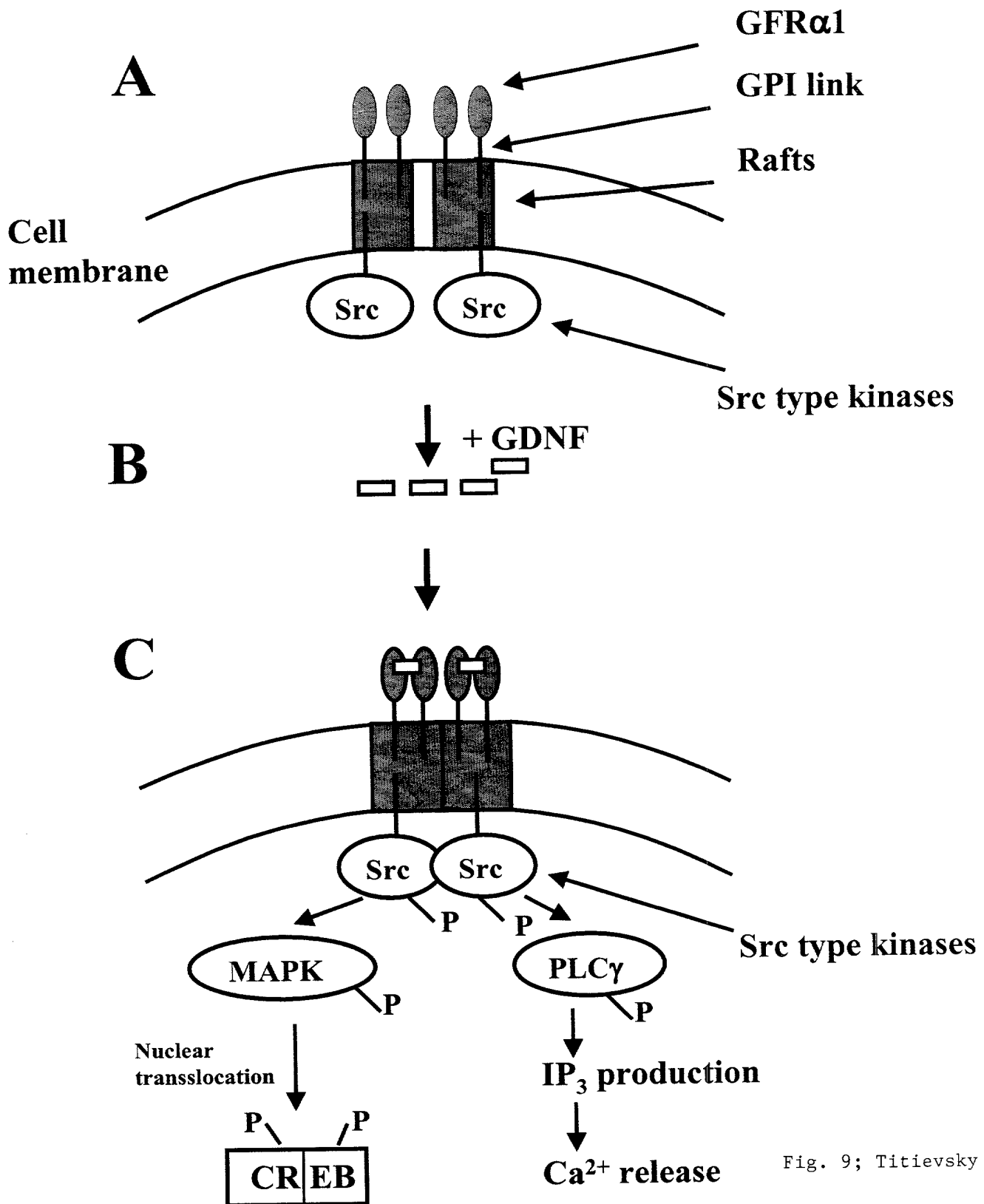


Fig.8; Titievsky et al.



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Alexey Vladimirovich Titievsky, Dmitri
Poteriaev, Urmas Arumäe and Mart Saarma

Group Art Unit: Not Yet
Assigned

For: A NOVEL RET-INDEPENDENT
SIGNALING PATHWAY FOR GDNF

Examiner: Not Yet Assigned

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

- ☒ is attached hereto.
☐ was filed on _____ as Serial No. _____
☐ said application having been amended on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
<input type="checkbox"/>	_____	_____	_____
<input type="checkbox"/>	_____	_____	_____

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned
_____	_____	_____
_____	_____	_____

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Serial Number	Date Filed
<u>60/102,647</u>	<u>October 1, 1998</u>
_____	_____

I hereby appoint the following persons of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 as attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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